Attorney Docket: NEX86/PCT-US



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| APPLICANT: | HICKE ET AL | | |
| SERIAL NO.: | 10/031,193 | EXAMINER: | FORMAN, B.J. |
| FILED: | JANUARY 31, 2002 | ART UNIT: | 1634 |
| TITLE: | TENACIN-C NUCLEIC ACID LIGANDS | CONFIRMATION NO. | 6209 |

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450



## APPEAL BRIEF

Sir:

In regard to the referenced application, Appellant submits this Appeal Brief.

### I. REAL PARTY IN INTEREST

The real party in interest is Gilead Sciences, Inc. The right of Gilead Sciences, Inc. to take action in the subject application was established by virtue of the following chain of title:

1. An assignment from the inventors to Gilead Sciences, Inc. is recorded at Reel 013120, Frame 0536.

### II. RELATED APPEALS AND INTERFERENCES

The undersigned legal representative of Appellant hereby confirms that there are no known appeals or interferences relating to the present application, or any parent application, which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

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**37 CFR 1.8**
**CERTIFICATE OF MAILING**

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Mail Stop Appeal Brief, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on June 6, 2005.

Signature: _____
Name: Denise S. Magee

## III. STATUS OF THE CLAIMS

Claims 33 and 44-58 are pending in the application. Claims 1-32 and 34-43 have been cancelled. No claims have been allowed. Claims 33 and 44-58 stand rejected under a final Office Action mailed September 7, 2004. The rejection of each of claims 33 and 44-58 is being appealed.

## IV. STATUS OF THE AMENDMENTS

In response to the Office Action of January 30, 2004, an amendment was filed on June 1, 2004. The claims set forth in section VIII reflect the claims pending as of the entry of the amendment of June 1, 2004.

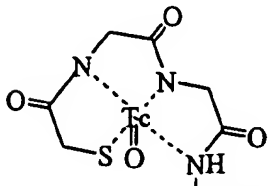
## V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 33 is drawn to a method for detecting the presence of a disease in a biological tissue, wherein the disease is characterized by the expression of tenascin-C in said tissue and wherein the disease is selected from the group consisting of cancer, psoriasis, and atherosclerosis. The method is comprised of attaching a marker that can be used in *in vivo* diagnostics to a tenascin-C nucleic acid ligand to form a marker-nucleic acid ligand complex; exposing the biological tissue which may contain the disease to the marker-nucleic acid ligand complex; and detecting the presence of the disease in the tissue by detecting the presence of the marker-nucleic acid ligand in the tissue. (Specification, page 7, lines 12-26).

Claims 44 and 56-58 further define the method of claim 33. Claim 44 is drawn to the method of claim 33 wherein the marker is selected from a radionuclide, fluorophore, magnetic compound or biotin (Specification, page 9, lines 26-32, page 14, lines 29-30). Claim 56 is drawn to method of claim 33 further comprising attaching a therapeutic or diagnostic agent to the complex (Specification, page 15, lines 13-19 and page 14, lines 21-27). Claim 57 is drawn to the method of claim 33 wherein the disease is cancer (Specification, page 14, line 32) and claim 58 is drawn to the method claim 33 wherein the tenascin-C nucleic acid ligand is identified using the SELEX method (Specification, page 14, lines 12-14).

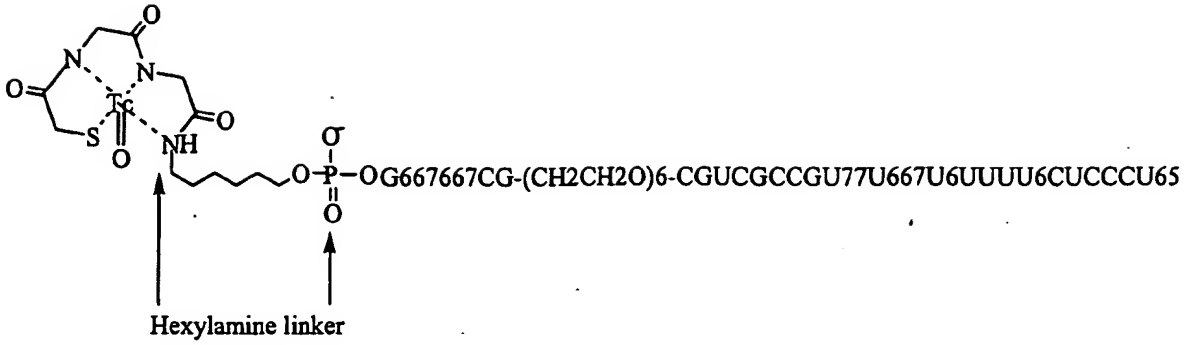
Claim 45 further defines claim 44. Claim 45 is directed to the method of claim 44 wherein the radionuclide is selected from technetium-99m (Tc-99m), Re-188, Cu-64, Cu-67, F-18, $^{125}I$, $^{131}I$, $^{111}In$, $^{32}P$ or $^{186}Re$ (Specification, page 9, lines 29-30) and claim 46 is directed to the method of claim 45 wherein the marker is technetium-99m (Specification, page 14, lines 28-29). Claim 47 is drawn to the method of claim 46 wherein the tenascin-C nucleic acid ligand comprises a linker (Specification, page 15, lines 6-7). Claims 48-50 further define claim 47. Claim 48 is drawn to the method of claim 47 wherein said linker is $(CH_2CH_2O)_6$ (Specification, page 10, lines 1-6 and Figure 2), claim 49 is drawn to the method of claim 47 wherein the linker is $NH_2(CH_2)_6OPO_3$ (Specification, page 10, lines 1-6, and Figure 2) and claim 50 is drawn to the method of 47 wherein the tenascin-C nucleic acid ligand is selected from the group consisting of SEQ ID NOS: 4-65 (Specification, Tables 3 and 4, pages 27-29).

Claim 51 is drawn to the method of claim 50 wherein said tenascin-C nucleic acid ligand is 5'-B-G667667CG-$(CH_2CH_2O)_6$-CGUCGCCGU77U667U6UUUU6CUCCCU65, wherein all pyrimidines are 2' F; 6 is 2'OMe G; 7 is 2' OMe A; 5 is 3'-3' dT and B is a linker. (Specification, page 22, lines 6-11). Claim 52 is drawn to the method of claim 51 wherein said technetium-99m is associated with a chelator (Specification, page 22, lines 22-24). Claim 53 is drawn to the method of claim 52, wherein the complex is:




B–G667667CG-(CH2CH2O)6-CGUCGCCGU77U667U6UUUU6CUCCCU65

(Specification, page 22, lines 22-24) and claim 54 is drawn to the method of claim 53 wherein the complex is




(Figure 10).

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 33 and 44-58 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

## VII. ARGUMENT

### A. The Rejection of claims 33 and 44-58 under 35 U.S.C. § 112, first paragraph

#### 1. Statement of the Relevant Law Pertaining to 35 U.S.C. § 112, first paragraph rejections.

The first paragraph of Section 112 requires that a patent application be written so as to "enable any person skilled in the art to which it pertains . . . to make and use the same." A specification is presumed to be enabling absent "a reason to doubt the objective truth of the statements contained therein." *In re Marzocchi*, 169 U.S.P.Q. 367, 369 (C.C.P.A 1971). A specification "may be enabling even though some experimentation is necessary," *United States v. Teletronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988), so long as the amount of experimentation required is not "undue experimentation." *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). The test is whether the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.*, 858 F.2d at 737. The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *Id.* The *Wands* court set forth a number of non-exclusive factors,

which a court may consider in determining whether a disclosure would require undue experimentation. These factors were set forth as follows:

(1) the quantity of experimentation necessary,

(2) the amount of direction or guidance presented,

(3) the presence or absence of working examples,

(4) the nature of the invention,

(5) the state of the prior art,

(6) the relative skill of those in the art,

(7) the predictability or unpredictability of the art, and

(8) the breadth of the claims.

Id.

**2. The Rejection of Claims 2-7 under 35 U.S.C. § 112, first paragraph is improper.**

Claims 33 and 44-58 have been rejected under 35 U.S.C. § 112, first paragraph for failure to comply with the enablement requirement. Specifically, the Examiner has stated that the specification does not enable one skilled in the art to which it pertains to make or use the invention commensurate with the scope of the claims. In support of this rejection the Examiner analyzes the claims in light of the factors set forth by the court in *In re Wands*.

Breadth of the Claims

With respect to the breadth of the claims, the Examiner finds that the specification teaches that tenascin-C is expressed in a variety of non-diseased tissues. In support of this statement, the Examiner refers to Figure 7 of the instant specification and states that this figure illustrates tenascin-C expression in liver, lung, spleen, intestine, and kidney. The Examiner further finds that the specification teaches only the detection of three specific xenografted tumor cell lines in mice with a single tenascin-C nucleic acid ligand, whereas the claims are broadly drawn to detecting cancer. In light of these teachings, the Examiner finds that the claims are unduly broad.

Applicant first notes that courts have determined that the scope of enablement must only bear a "reasonable correlation" to the scope of the claims. *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (C.C.P.A. 1970).

Applicants respectfully disagree with the assertion that the specification teaches the expression of tenascin-C in non-diseased tissues. At that the time of filing the application, it was well known in the art that tenascin-C is expressed during embryogenesis. In adults, it expressed during wound healing, neoplasia, hyperproliferative skin disorders (such as psoriasis) and in atherosclerosis. See page 1, lines 17-32 where there is an extensive discussion of the prior art teaching that tenascin-C is expressed exclusively in such diseased tissue. In the case of tumors, tenascin-C expression is predictive of local tumor recurrence and is correlated with invasiveness and distant metastasis. See for example, Jahkola *et al.*, *Eur. J. Cancer* 34:1687-1692 (1998); Ishihara *et al.*, *Clin. Cancer Res.* 1:1035-1041 (1995); Jahkola *et al.*, *Br. J. Cancer* 78:1507-1513 (1998).

Figure 7, contrary to the Examiner's assertion, does not teach otherwise. Figure 7 illustrates the biodistribution of $^{111}$In-DOTA or $^{111}$In-DTPA radiolabeled tenascin-C aptamer. DOTA and DPTA are the chelators used to conjugate the $^{111}$In radiolabel to the aptamer. It can be seen in Figure 7 that $^{111}$In label is observed in liver, spleen, and kidney. It was well known in the art at the time the instant application was filed that the chelating agent used in the preparation of radiopharmaceuticals can affect the biodistribution of the radiopharmaceutical in a living animal. For example, different chelators can cause the non-specific uptake of radiopharmaceuticals to the kidneys, intestine, hepatobiliary system and lungs, even when the radiopharmaceutical comprises a specific targeting agent--such as a monoclonal antibody or aptamer--that would not be expected to bind in those regions. Such distributions are well known in the art as the inevitable consequence of administering an agent to living, metabolizing organism with a circulatory system. One skilled in the art realizes that after waiting an appropriate period to allow this normal tissue clearance of radioactivity to occur, the patient may then be imaged, and tumors are identified by defining regions of the body with increased tracer uptake. See, for example, Bast *et al.*, *Cancer Medicine*, 5th Edition, § 16, Part 65 (2000) (available online at << http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books>> ) where the authors state:

> In radioimmunoscintography, a tumor-specific antibody is labeled with a radionuclide that can be visualized outside the body by nuclear medicine imaging methods. After waiting an appropriate period for normal tissue clearance of radioactivity to occur, the patient is imaged, and tumors are identified by defining regions of the body with increased tracer uptake. ...

See also Hjelstuen, *Analyst.* 120: 863-866 (1995). Indeed, the instant specification teaches at page 23, lines 29-32:

> Tc-99m radioactivity also appears in other tissues, notably the small and large intestines. The hepatobiliary clearance pattern seen here can be readily altered by one skilled in the art, for example by altering the hydrophilicity of the Tc-99m chelator, changing the chelator, or changing the radiometal/chelator pair together.

Similarly, at page 25, lines 23-29 (referring to Figure 7):

> This experiment indicates that the chemical properties of the chelator have a large effect on the distribution of the radiolabel of TTA1/GS7641 within a living animal. Biodistribution patterns that are different from Hi15-TTA1/GS7641 may be useful for targeting tumors under certain conditions where hepatobiliary clearance is undesired. Such conditions include, but are not limited to radiotherapy applications as well as imaging of the intestines, prostate and other abdominal regions.

Thus, one skilled in the art at the time the application was filed would appreciate that the presence of the radiolabel in the normal tissues mentioned by the examiner does not indicate that those tissues express tenascin-C. Moreover, one skilled in the art, guided both by the prior art at the time of filing and the specific teachings of the instant specification quoted above, would be able to discriminate between tumor specific expression of tenascin-C and the non-specific presence of the label in certain tissues as a result of hepatobiliary clearance. For example, see Figure 3 of the instant specification where tumors are clearly visible despite the additional radioactivity in the gastrointestinal tract. Finally, the specification teaches that one may alter a hepatobiliary clearance pattern by altering the hydrophilicity of the chelator, changing the chelator, or changing the radiometal/chelator pair together. For example, the specification teaches that if one wants to perform abdominal imaging, then one selects a chelator that has a relatively low level of hepatobiliary clearance. See page 25, lines 23-39.

Turning now to the Examiner's finding that the specification teaches only detection of a few types of cancer using a single tenascin-C nucleic acid ligand whereas the claims are drawn broadly to the detection of cancer. Applicant respectfully submits that there is a reasonable

correlation between the scope of the claims and the enabling disclosure of the specification. Applicants have provided examples of the detection of widely varied types of human tumors xenografted into mice including (with reference to the cell types listed in Figure 6):

| | |
|---|---|
| neural tumors | U87 glioblastoma-astrocytoma<br>U251 glioma |
| breast tumors | MDA-MB-468 poorly metastatic breast cancer<br>MDA-MB-435 highly metastatic breast cancer |
| colorectal tumors | SW620 colonic carcinoma |
| soft tissue sarcoma | A673 rhabdomyosarcoma |

The prior art is replete with descriptions of the utility of the mouse xenograft model in the development of methods for the detection and treatment of human tumors, indicating that the mouse xenograft model is recognized in the art as an *in vivo* model that correlates well with human tumors. For example, see Rofstad & Lyng, *Mol Med Today.* 2:394-403 (1996) (xenograft model systems for human melanoma); Clarke, *Breast Cancer Res Treat.* 39:69-86 (1996) (xenograft model systems for breast cancer ); Thompson *et al.*, *Biochim Biophys Acta.* 1400(1-3):301-19 (1998) (describing success of mouse xenograft model system in developing cancer therapeutics). In Bast *et al.*, *Cancer Medicine*, 5th Edition, § 13, Part 42 (2000), the authors state:

> The success of human tumor xenografting into the nude mice and the ability to maintain the histologic and biologic identity of tumors through successive passages in vivo revolutionized many aspects of cancer research, including drug development. Transplantation of tumor cell lines into nude mice can be accomplished via multiple routes: subcutaneous, intraperitoneal, intravenous, intracranial, intrasplenic, renal subcapsular, or through a new orthotopic model by site-specific organ inoculation. Each site has specific advantages and limitations.
>
> . . .
>
> Despite these changes in kinetics of invasive potential, the majority of the xenografted human tumors maintain the morphologic and biochemical characteristics of their original tumors. Therefore, it is expected that chemosensitivity would be similar in both the original and the xenografted human tumor, and that this correlation would predict for both active single agents and active drug combinations. In fact, excellent correlations can be made between

> average growth delay for human tumors in nude mice treated with the best available drug combinations and complete clinical response rates. ...

With respect to tenascin-C specifically, there are numerous reports of the use of the xenograft tumor model in combination with radiolabeled monoclonal antibodies. For example, Bourdon *et al.*, *Anticancer Res.* 4(3):133-140 (1984) describe the use of the anti-tenascin antibody 81C6 to image human tumor xenografts in mice. Indicating that such studies of the 81C6 antibody correlate well with studies in humans, Schold *et al.*, *Invest Radiol.* 28(6):488-496 (1993) and Zalutsky *et al.*, *Cancer Res.* 49(10):2807-2813 (May 1989) describe the use of radiolabeled 81C6 to image tumors in human patients; and Reardon *et al.*, *J Clin Oncol.* 20(5):1389-1397 (2002) describe the use of 81C6 to treat human tumors.

The prior art includes the descriptions of many types of tumors that express tenascin-C (including carcinomas of the lung, breast, prostate, colon, astrocytomas, glioblastomas, melanomas, and sarcomas), as well as other diseases such as hypoproliferative skin disorders (e.g., psoriasis). See page 1, line 17- page 2, line 1 of the instant specification where such descriptions are incorporated by reference. At the time the instant application was filed, tenascin-C had additionally been implicated in numerous other neoplasms, including: basal cell carcinoma (Stamp, *J Pathol.* 159(3):225-229 (Nov. 1989)), odontogenic tumors (Heikinheimo *et al.*, *Virchows Arch B Cell Pathol Incl Mol Pathol.* 61(2):101-109 (1991)), endometrial adenocarcinoma (Vollmer *et al.*, *Lab Invest.* 62(6):725-730 (Jun 1990)), hepatocellular carcinoma (Yamada *et al.*, *Liver* 12(1):10-16 (Feb. 1992)), salivary gland tumors (Soini *et al.*, *Virchows Arch A Pathol Anat Histopathol.* 421(3):217-222 (1992)), transitional cell carcinomas of the bladder (Tiitta *et al.*, *Virchows Arch B Cell Pathol Incl Mol Pathol.* 63(5):283-287 (1993)), and in rhabdomyosarcomas, fibromas and liposarcomas (Schnyder *et al.*, *Int J Cancer.* 72(2):217-224 (Jul 17,1997)).

In summary, the prior art recognizes that tenascin-C is expressed in a tremendous variety of human tumors, and in hyperproliferative skin disorders and atherosclerosis. As explained above, Figure 7 in the instant specification does not demonstrate extensive expression in normal tissue. The prior art also recognizes the general utility of the mouse xenograft model for the study of human tumors. Finally, the prior art recognizes that the specific utility of the mouse

xenograft model for the study of human tumors expressing tenascin-C. Thus, Applicants submit that by providing actual reduction to practice of the detection of xenografted human tumors of widely diverse origins, the scope of enablement reasonably correlates with the scope of the claims.

Nature of the Invention

The Examiner submits that the nature of the invention is such that detecting a disease using a ligand would require a teaching of a relationship between the ligand and the disease wherein the teaching would include an illustration or examples of the relationship between the ligand and the disease. As an example, the Examiner suggests a sample population study illustrating that tenascin-C expression detects cancer regardless of the amount, time, or pattern of expression.

As detailed in the foregoing section of this response, the state of the art at the time of filing of the invention was such that it was known that tenascin-C is expressed during embryogenesis, and during certain disease processes in adults, including cancer, proliferative skin disorders and atherosclerosis. See page 1, lines 17-32 of the instant specification where there is discussion of the types of diseased tissue where tenascin-C is expressed. The prior art also teaches that radiolabeled antibodies that bind tenascin-C are used for imaging and therapy of tumors in a clinical setting. See page 1, line 33-page 2, line 1. In addition, the specification provides examples in which tenascin-C aptamer recognizes a variety of morphologically distinct human tumors that have been xenografted into mice. Thus, the specification provides a teaching of the clear relationship between tenascin-C expression and disease.

As also explained in the foregoing section of this response, Figure 7 of the application does not depict the expression of tenascin-C in non-diseased tissues. Rather, Figure 7 depicts the inevitable hepatobiliary clearance pattern of a radiopharmaceutical in a living animal. Such hepatobiliary clearance patterns are well known in the radiopharmaceutical art. The specification teaches that one may alter a hepatobiliary clearance pattern by altering the hydrophilicity of the chelator, changing the chelator, or changing the radiometal/chelator pair together. See page 23, lines 29-32. For example, the specification teaches that if one wants to perform abdominal imaging, then one selects a chelator that has a relatively low level of hepatobiliary clearance. See page 25, lines 23-39.

Not in claim

For the foregoing reasons, Applicant respectfully submits there is a clear teaching of the relationship between tenascin-C expression and the diseases of cancer, psoriasis and atherosclerosis which would enable one skilled in the art to make and use the invention as claimed.

Level of Predictability in the Art

The Examiner states that the level of predictability in the art is very low with regard to detection of disease without a correlating relationship between the disease and the detecting molecule. The Examiner also states that the relationship between tenascin-C expression and cancer, psoriasis or atherosclerosis is unknown and that Figure 7 of the specification illustrates the expression of tenascin-C in normal tissue.

As discussed in detail above, the prior art clearly teaches a correlating relationship between expression of tenascin-C and cancer, psoriasis and atherosclerosis. See specification, page 1, lines 17-32. The prior art also teaches that radiolabeled antibodies that bind tenascin-C are used for imaging and therapy of tumors in a clinical setting. See page 1, line 33-page 2, line 1. Thus, the specification provides a teaching of the clear relationship between tenascin-C expression and disease.

In addition, Figure 7 of the application does not depict the expression of tenascin-C in non-diseased tissues. Rather, Figure 7 depicts the inevitable hepatobiliary clearance pattern of a radiopharmaceutical in a living animal. Such hepatobiliary clearance patterns are well known in the radiopharmaceutical art. The specification teaches that one may alter a hepatobiliary clearance pattern by altering the hydrophilicity of the chelator, changing the chelator, or changing the radiometal/chelator pair together. See page 23, lines 29-32. For example, the specification teaches that if one wants to perform abdominal imaging, then one selects a chelator that has a relatively low level of hepatobiliary clearance. See page 25, lines 23-39.

For these reasons, one skilled in the art would readily extrapolate the prior art teachings--that tenascin-C expression correlates with disease and that tenascin-C antibodies may be used for therapy and detection of those diseases--to the claimed invention. MPEP § 2164.03. Therefore, contrary to the Examiner's assertion the level of predictability in the art is high with regard to detecting tenascin-C in order to detect a disease.

Existence of Working Examples

The Examiner asserts that the specification does not provide working examples of the broadly claimed invention, stating that the specification teaches the expression of tenascin-C in a variety of non-diseased tissues. Applicants respectfully disagree.

It has been determined by the courts that no working examples are required to enable a patent application. *In re Borkowski*, 422 F.2d 904, 164 U.S.P.Q. 642 (C.C.P.A. 1970). Applicant, however, has provided a number of specific *in vivo* examples of the detection of widely varying types of human tumors using the art-recognized mouse xenograft model. The state of the art is such that the mouse xenograft model for tumor detection is accepted as reasonably correlating with the detection of human tumors *in vivo*. Note that a rigorous or invariable exact correlation is not required. *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739, 747 (Fed. Cir. 1985). As discussed above in detail, the prior art recognizes both the general utility of the mouse xenograft model for the study of human tumors and the *specific* utility of the mouse xenograft model for the study of human tumors expressing tenascin-C. For example, Bourdon *et al.*, *Anticancer Res.* 4(3):133-140 (1984) describe the use of the anti-tenascin antibody 81C6 to image human tumor xenografts in mice. Indicating that such studies of the 81C6 antibody correlate well with studies in humans, Schold *et al.*, *Invest Radiol.* 28(6):488-496 (1993) and Zalutsky *et al.*, *Cancer Res.* 49(10):2807-2813 (May 1989) describe the use of radiolabeled 81C6 to image tumors in human patients; and Reardon *et al.*, *J Clin Oncol.* 20(5):1389-1397 (2002) describe the use of 81C6 to treat human tumors.

Contrary to the Examiner's assertion, Figure 7 of the application does not depict the expression of tenascin-C in non-diseased tissues. Rather, Figure 7 depicts the inevitable hepatobiliary clearance pattern of a radiopharmaceutical in a living animal. Such hepatobiliary clearance patterns are well known in the radiopharmaceutical art. The specification teaches that one may alter a hepatobiliary clearance pattern by altering the hydrophilicity of the chelator, changing the chelator, or changing the radiometal/chelator pair together. See page 23, lines 29-32 . For example, the specification teaches that if one wants to perform abdominal imaging, then one selects a chelator that has a relatively low level of hepatobiliary clearance. See page 25, lines 23-39.

In summary, the specification provides examples of the detection of widely varied human tumors using the art-recognized mouse xenograft model. Applicant submits that the Examiner's

focus on the difference between the working examples and the claimed methods is based on a standard of a rigorous or exact correlation, which is not the standard of enablement. The prior art recognizes that tenascin-C is an important marker for the detection of diseased tissue, including cancer, psoriasis and atherosclerosis (see page 1, lines 17-33 of the instant specification). Figure 7 of the application is not to the contrary. The prior art also recognizes that detection of tenascin-C in human tumors in xenografted mice correlates with the detection of tenascin-C expression in tumors in humans. For these reasons, Applicants respectfully submit that the specification provides working examples of the claimed invention, which would enable one skilled in the art to make and use the invention as claimed.

Quantity of Experimentation Required

The Examiner submits that it would require undue experimentation for one skilled in the art to make and use the invention as claimed in view of the breadth of the claims, the nature of the invention, the unpredictability in the art, and the lack of working examples. Applicants respectfully disagree. As detailed in the foregoing sections of the response, Applicants have demonstrated that the art recognizes the correlation between tenascin-C expression and cancer, psoriasis and atherosclerosis. Applicants have also demonstrated that the art recognizes the correlation between the detection and treatment of human tumors xenografted into mice with the detection and treatment of humans with the same tumors. In the specific case of tumors that express tenascin-C, Applicants have demonstrated that the art recognizes the correlation between the detection of tenascin-C expression in xenografted mice and the detection and therapy of the same tumors in humans. Applicants have demonstrated that a representative tenascin-C nucleic acid ligand detects a wide variety of human tumors in xenografted mice.

While development of a specific treatment or detection regimen may require a large quantity of experimentation, the amount of experimentation is not a controlling factor. It is a tenet of patent law that an applicant need not teach what the skilled artisan already knows. Instead, it is preferred that an applicant "omit what is known in the art." *Hybritech Inc. v. Monoclonal Antibodies*, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). Certainly, much guidance and direction exists in the prior art with regard to administration routes and other details of treatment regimens. Antisense and other nucleic acid therapeutics have been used in the treatment of humans since at least as early as 1993. Additionally, the specification provides extensive

teaching of the preparation, administration, and analysis of treatment data in *in vivo* experiments. Applicants submit that such prior art treatments, together with the teachings of the specification, provide a reasonable amount of guidance with respect to the direction in which the experimentation should proceed and, and therefore satisfy the enablement requirement.

## VIII. CLAIMS APPENDIX

1-32 (Canceled).

33 (Previously Presented). A method for detecting the presence of a disease in a biological tissue which may contain said disease, wherein said disease is characterized by the expression of tenascin-C in said tissue and wherein said disease is selected from the group consisting of cancer, psoriasis, and atherosclerosis, the method comprising:

a) attaching a marker that can be used in in vivo diagnostics to a tenascin-C nucleic acid ligand to form a marker-nucleic acid ligand complex;

b) exposing said biological tissue which may contain said disease to said marker-nucleic acid ligand complex; and

c) detecting the presence of said disease in said tissue by detecting the presence of said marker-nucleic acid ligand in said tissue.

34-43 (Canceled).

44 (Previously Presented). The method of 33 wherein said marker is selected from the group consisting of radionuclides, fluorophores, magnetic compounds, and biotin.

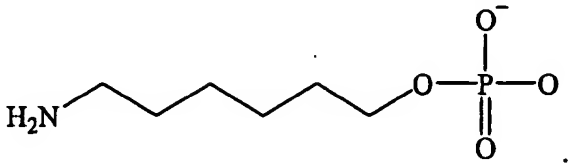
45 (Previously Presented). The method of 44 wherein said radionuclide is selected from the group consisting of technetium-99m (Tc-99m), Re-188, Cu-64, Cu-67, F-18, $^{125}I$, $^{131}I$, $^{111}In$, $^{32}P$, and $^{186}Re$.

46 (Previously Presented). The method of 45 wherein said marker is technetium-99m.

47 (Previously Presented). The method of 46 wherein said tenascin-C nucleic acid ligand comprises a linker.

48 (Previously Presented). The method of 47 wherein said linker is $(CH_2CH_2O)_6$.

49 (Previously Presented). The method of 47, wherein said linker has the structure



50 (Previously Presented). The method of 47 wherein said tenascin-C nucleic acid ligand is selected from the group consisting of SEQ ID NOS: 4-65.

51 (Previously Presented). The method of 50 wherein said tenascin-C nucleic acid ligand is

5'-B-G667667CG-$(CH_2CH_2O)_6$-CGUCGCCGU77U667U6UUUU6CUCCCU65

wherein:

all pyrimidines are 2' F;

6= 2'OMe G;

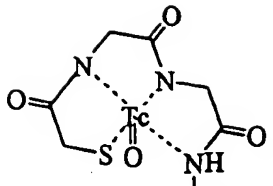
7= 2' OMe A;

5= 3'-3' dT; and

B= linker.

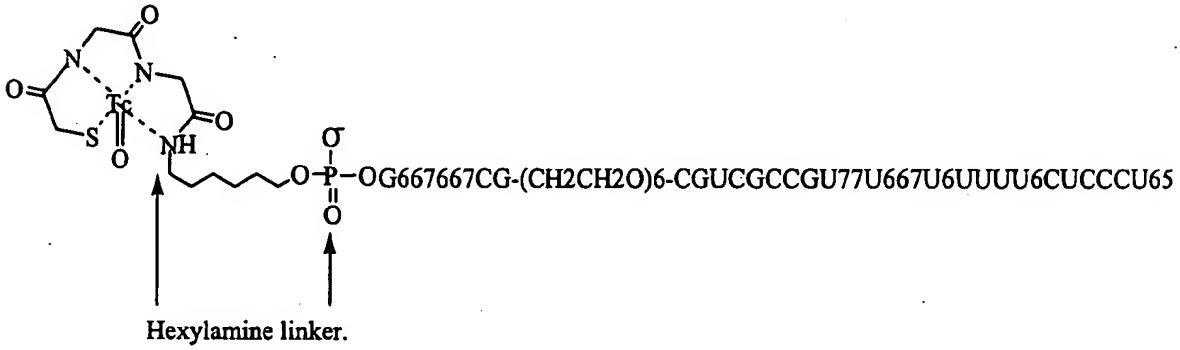
52 (Previously Presented). The method of 51 wherein said technetium-99m is associated with a chelator.

53 (Previously Presented). The method of 52, wherein said complex is



B–G667667CG-(CH2CH2O)6-CGUCGCCGU77U667U6UUUU6CUCCCU65

54 (Previously Presented). The method of 53 wherein said complex is




55 (Canceled).

56 (Previously Presented). The method of 33 further comprising attaching a therapeutic or diagnostic agent to said complex.

57 (Previously Presented). The method of 33 wherein said disease is cancer.

58 (Previously Presented). The method of 33 wherein said tenascin-C nucleic acid ligand is identified by:

i) contacting a candidate mixture of nucleic acids with tenascin-C wherein nucleic acids having an increased affinity to tenascin-C relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

ii) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture;

iii) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids with relatively higher affinity and specificity for binding to tenascin-C, whereby a nucleic acid ligand of tenascin-C is identified.

## IX. EVIDENCE APPENDIX

Enclosed please find copies of the following references of record in this appeal:


1. Jahkola *et al.*, Eur. J. Cancer 34:1687-1692 (1998)
2. Ishihara *et al.*, Clin. Cancer Res. 1:1035-1041 (1995)
3. Jahkola *et al.*, Br. J. Cancer 78:1507-1513 (1998)
4. Bast *et al.*, *Cancer Medicine*, 5th Edition, § 16, Part 65 (2000)
5. Hjelstuen, *Analyst.* 120: 863-866 (1995)
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7. Clarke, *Breast Cancer Res Treat.* 39:69-86 (1996)
8. Thompson *et al.*, *Biochim Biophys Acta.* 1400(1-3):301-319 (1998)
9. Bast *et al.*, *Cancer Medicine*, 5th Edition, § 13, Part 42 (2000)
10. Bourdon *et al.*, *Anticancer Res.* 4(3):133-140 (1984)
11. Schold *et al.*, *Invest Radiol.* 28(6):488-496 (1993)
12. Zalutsky *et al.*, *Cancer Res.* 49(10):2807-2813 (May 1989)
13. Reardon *et al.*, *J Clin Oncol.* 20(5):1389-1397 (2002)
14. Stamp, *J Pathol.* 159(3):225-229 (Nov. 1989)
15. Heikinheimo *et al.*, *Virchows Arch B Cell Pathol Incl Mol Pathol.* 61(2):101-109 (1991)
16. Vollmer *et al.*, *Lab Invest.* 62(6):725-730 (Jun 1990)
17. Yamada *et al.*, *Liver* 12(1):10-16 (Feb. 1992)
18. Soini *et al.*, *Virchows Arch A Pathol Anat Histopathol.* 421(3):217-222 (1992)
19. Tiitta *et al.*, *Virchows Arch B Cell Pathol Incl Mol Pathol.* 63(5):283-287 (1993)
20. Schnyder *et al.*, *Int J Cancer.* 72(2):217-224 (Jul 17,1997)

## X. CLOSING REMARKS

For the foregoing reasons, Appellant submits that the lack of enablement of claims 33 and 44-58 has not been established, and that the claims are therefore patentable. Enclosed is a check in the amount of $500.00 to cover the cost of the fee for this Appeal Brief. It is believed that no other fees are due with this Appeal Brief. If this is in error, please charge any additional fees to Deposit Account No. 19-5117.

Respectfully submitted,

Date: June 6, 2005

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## Original Paper

# Expression of Tenascin-C in Intraductal Carcinoma of Human Breast: Relationship to Invasion

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**Tenascin-C (Tn-C) is an extracellular matrix glycoprotein that appears in areas of epithelial-mesenchymal interaction during fetal development and in neoplasia. The immunohistochemical expression of Tn-C and its relationship to histology, nuclear grade, microinvasion, oestrogen (ER) and progesterone receptors (PR), and to cell proliferation measured by Ki-67 expression were studied in 89 intraductal breast carcinomas (DCIS). Periductal Tn-C was noted in 87% and stromal Tn-C in 25% of the tumours. Stromal expression was associated with moderate to strong periductal expression and microinvasion. Periductal expression was associated with comedo-type, nuclear grade, microinvasion, Ki-67 expression, and lack of PR. The distribution of Tn-C was compared in DCIS and in the intraductal component from another series of small axillary node-negative invasive breast carcinomas ($n = 44$). Tn-C was present in the stroma of pure DCIS in 25% and in the intraductal component of the other series in 82%. Thus, stromal or moderate to strong periductal Tn-C expression in DCIS may relate to early invasion. DCIS with weak periductal or missing Tn-C expression may be a subgroup with benign behaviour.** © **1998 Elsevier Science Ltd. All rights reserved.**

**Key words: tenascin, intraductal carcinoma, breast cancer, invasion**
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## INTRODUCTION

CARCINOMAS ARE composed of two discrete but interdependent compartments, malignant cells and the stroma [1]. The stroma differs from normal connective tissue and resembles the aggregations of mesenchyme observed during morphogenesis [2] or the granulation tissue that forms during wound healing [1]. Tenascin-C (Tn-C) is a large glycoprotein of the extracellular matrix, expressed transiently during fetal development, inflammation, wound healing and neoplasia. It is believed to have active functions in epithelial-mesenchymal interactions. Cell culture studies suggest that it has growth-promoting activity and anti-adhesive functions (for a review see [3]). Tn-C is produced by stromal fibroblasts, and also by epithelial cells of normal and malignant breast tissues, as shown by *in situ* hybridisation techniques [4, 5]. When expressed in normal adult breast tissue, Tn-C is located immunohistochemically as a continuous thin layer around the ducts. In intraductal carcinoma (ductal/carcinomas *in situ*, DCIS), Tn-C appears as broad bands and, in infiltrating ductal carcinomas, extensive immunostaining is noted in the stroma around clusters of carcinoma cells [6]. However, not all breast carcinomas express Tn-C [7, 8]. The reaction is most intense at the invasive edge of the tumour [6]. Tn-C may indicate the site of active cancer spread, since expression of Tn-C in the invasion border of small breast carcinomas is associated with an adverse patient outcome [9, 10].

DCIS is a precursor lesion of breast carcinoma. The epithelial cells are malignant, but growth is limited to the inside of the gland ducts by a basement membrane [11]. The comedo DCIS (which always presents with central necrosis) is known to be associated with occult foci of invasion more often than the other histological subtypes of DCIS [11] and the rate of local recurrences in the comedo type is also higher [12, 13]. Diagnostic classifications of DCIS, based on the nuclear grade and the presence or absence of central necrosis,

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have been proposed [12–14]. The search for treatment criteria for DCIS has started and in 1997 at least three prospective multicentre studies were under way to find out who should be treated with resection only, who needs postoperative radiotherapy and who should undergo mastectomy [15].

In the present study, the immunohistochemical distribution of Tn-C was analysed in tissue from 89 DCIS, with the aim of characterising the staining pattern and intensity of Tn-C and relating the results to subtype, nuclear grade and microinvasion. The level of Ki-67 antigen as a measure of proliferation activity and of oestrogen receptors (ER) and progesterone receptors (PR) had been determined previously in the majority of these tumours and the results are included in the study. The distribution of Tn-C expression was also compared in this group of pure DCIS and in the intraductal component areas of material from another 44 small invasive tumours.

## PATIENTS AND METHODS

*Patients*

133 patients, were operated upon for primary DCIS during 1974–1996 at the Fourth Department of Surgery, Helsinki University Central Hospital (HUCH), Helsinki, Finland. For this study, the pathology reports and specimens of these 133 tumours were reviewed and the most representative sample of each case was selected for Tn-C immunohistochemistry. In 36 cases, either no tumour sample was available or no malignant tissue was left to investigate. A pre-operative core-needle biopsy had been taken of 16 tumours and these cases were excluded to avoid misinterpretation of possible stromal expression of Tn-C related to trauma [16]. The remaining 89 tumours were included in this study.

The median age of the patients was 52.5 years (range 28–82 years). 39 women (44%) had a breast-conserving operation and 28 of these (72%) received postoperative radiotherapy of 50 Gy to the breast area. No lymph node metastases were found in the 26 women (29%) who had an axillary dissection. The follow-up data were collected in November 1997 from the records of the Fourth Department of Surgery and the Department of Oncology, HUCH and the Finnish Cancer Registry.

Another group of 44 patients with small (1–25 mm) axillary node-negative tumours, comprised of both an invasive area and an intraductal component, has been characterised in detail previously [9].

*Tumour samples*

The tumours were re-examined and classified for histological subtype and nuclear grade. Nuclear grade was classified as low (1), intermediate (2) and high (3) [17]. The largest dimension of the tumours was obtained from the pathology reports. The mean size was 15 mm (range 2–40 mm). In 26 tumours (29%), the pathologist had not reported the size of the tumour, apparently because of widespread multifocality. The paraffin-embedded tumours were cut at 5 $\mu$m for Tn-C immunohistochemistry, and the sections were also stained with haematoxylin–eosin to verify histology.

Steroid receptors and Ki-67 were routinely determined in the laboratory, although in the earlier specimens these determinations were missing. No retrospective staining was carried out. ER was available in 64 (72%), PR in 63 (71%) and Ki-67 antigen in 61 (69%) of the tumours.

*Tn-C immunohistochemistry*

The monoclonal antibody 143BD7 against Tn-C has been previously characterised [18] and the immunohistochemical detection and evaluation of Tn-C has been described [9]. The extent and intensity of Tn-C expression was scored as −, +, ++ and +++ corresponding to negative, weak, moderate, and strong immunoreactivity. Tn-C staining was scored for periductal and stromal expression.

*ER and PR immunohistochemistry*

Until October 1995, the steroid receptors were assessed in frozen sections. Frozen sections were used for determinations of ER in 46 tumours and of PR in 45 tumours. Immunohistochemistry was performed using the ERICA and PRICA kits according to the instructions of the manufacturer (Abbott Laboratories, Chicago, Illinois, U.S.A.). The result was scored as weakly positive (+) when 10–40% of cells were stained, as moderately positive (++) when 40–70% were stained, and as strong (+++) when >70% were stained. The staining intensity was not recorded. After October 1995 the staining was performed on paraffin-embedded sections. The tissue arrived fresh to the laboratory and the tumour specimen was immediately fixed in formaldehyde. Steroid receptors were determined from paraffin sections of 18 tumours using the monoclonal antihuman ER antibody (clone 1D5, Dako, Glostrup, Denmark) and PRICA kit for PR. The staining method for PR has been described previously [19]. ER was detected using the same microwave pretreatment as for PR, but the detection was carried out with a commercial ABC kit (Vectastain Elite, Vector Laboratories, Burlingame, California, U.S.A.). The scoring was the same as in frozen sections.

*Ki-67 immunohistochemistry*

Ki-67 immunohistochemistry was performed using monoclonal Ki-67 antiserum (Dakopatts) on frozen tissue in 21 tumours before February 1994 (for methods, see [20]) and thereafter, using the polyclonal Ki-67 antibody (Dako) on paraffin-embedded tissue in 40 tumours (for methods, see [21]). The level of immunoreactivity was expressed as the proportion of Ki-67 positive cells. The intensity of the staining was not recorded. The frozen sections were scored as weakly positive (+) when 1–2% of the nuclei were positive, as moderate (++) when 3–10% of the nuclei were positive and as strong (+++) when >10% of the nuclei were positive. For the paraffin-embedded tumour material, the corresponding proportions of Ki-67-positive nuclei were 5–15% (+), 15–30% (++) and >30% (+++).

*Statistical methods*

The statistical significance of differences in periductal and stromal Tn-C distribution between the intraductal component of invasive cancers and pure DCIS was tested with the chi-square test. The chi-square test was also used to test for an association between discrete variables and the Mann–Whitney $U$ test for continuous variables. All tests were two-sided and $P$ values below 0.05 were considered significant.

## RESULTS

*Expression of Tn-C in DCIS*

Tn-C expression was observed periductally in 77 tumours (87%). It was weak in 23 (26%), moderate in 29 (33%) and strong in 25 tumours (28%). In some samples, Tn-C was not

present around all the affected ducts. In a few cases, periductal staining was also seen around benign hyperplastic ducts. When stromal Tn-C was encountered with microinvasion, it seemed to accompany the invading cells (Figure 1). Typically, the stromal Tn-C was focal, often forming bridges between the ducts of DCIS. In two cases, stromal Tn-C was expressed in areas of benign fibrotic stroma. Stromal expression was absent in 67 tumours (75%), but weak in nine (10%), moderate in nine (10%) and strong in four (5%). For analysis of associations with other factors, the stromal Tn-C-positive groups were combined ($n = 22$).

*Expression of ER and PR in DCIS*

In the 64 tumours studied, 20 (31%) were ER-negative tumours, four (6%) weakly positive, eight (13%) moderately positive and 32 (50%) strongly positive. For PR ($n = 63$) 32 (51%) were negative, five (8%) were weakly positive, 12 (19%) were moderately positive and 14 (22%) strongly positive. For analysis, the steroid receptors were dichotomised to positive and negative groups.

*Expression of Ki-67 in DCIS*

Of the 61 tumours studied, 18 (30%) were Ki-67 negative, 24 (39%) weakly positive, 13 (21%) moderately positive and six (10%) strongly positive. For analysis, the Ki-67-positive groups were combined ($n = 42$).

*Relationship of Tn-C to other variables*

Histological classification and Tn-C staining of the tumours are shown in Table 1. Only two of the tumours (2%) were of low grade, 29 (33%) of intermediate grade and 58 (65%) of high grade. In mixed types, the one with the worse

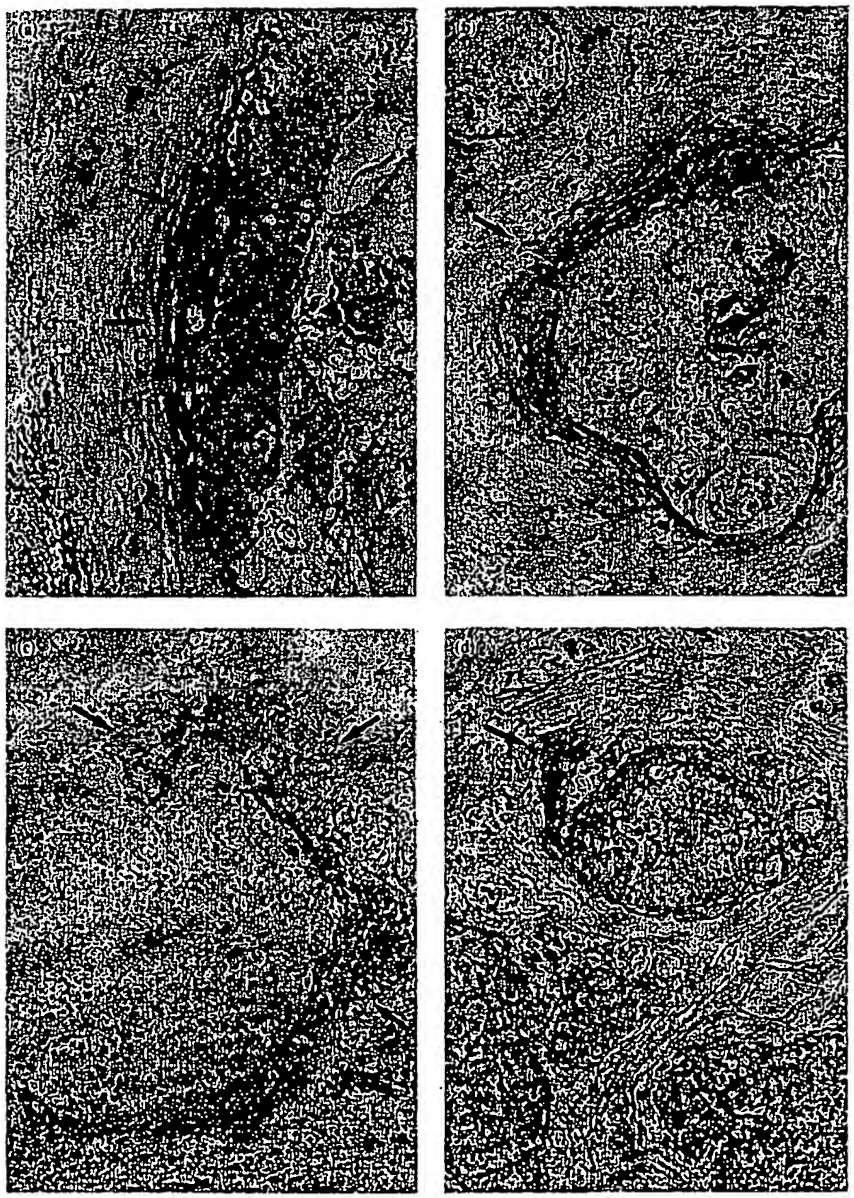

**Figure 1.** Periductal tenascin-C expression in four tumours of intraductal carcinomas (a–d). Note focally enhanced expression in microinvasive foci (arrows in a–d). (magnification×125).

*Table 1. Distribution of periductal and stromal tenascin-C (Tn-C) immunoreactivity in intraductal carcinoma (DCIS) and subtypes*

| | Periductal | | | | Stromal | | | |
|---|---|---|---|---|---|---|---|---|
| | – n (%) | + n (%) | ++ n (%) | +++ n (%) | – n (%) | + n (%) | ++ n (%) | +++ n (%) |
| All DCIS ($n=89$) | 12 (13) | 23 (26) | 29 (33) | 25 (28) | 67 (75) | 9 (10) | 9 (10) | 4 (5) |
| Comedo ($n=47$) | 1 (2) | 12 (26) | 15 (32) | 19 (40) | 35 (74) | 7 (15) | 4 (9) | 1 (2) |
| Non-comedo ($n=42$) | 11 (26) | 11 (26) | 14 (33) | 6 (14) | 32 (76) | 2 (5) | 5 (12) | 3 (7) |
| Cribriform ($n=12$) | 2 (17) | 1 (8) | 6 (50) | 3 (25) | 6 (50) | 2 (17) | 3 (25 | 1 (8) |
| Micropapillary ($n=4$) | 2 (50) | 1 (25) | 1 (25) | 0 | 4 (100) | 0 | 0 | 0 |
| Not specified ($n=26$) | 7 (27) | 9 (35) | 7 (27) | 3 (12) | 22 (84) | 0 | 2 (8) | 2 (8) |

*Table 2. Associations between periductal tenascin-C (Tn-C) expression and other variables of intraductal carcinoma (DCIS)*

| | Periductal Tn-C expression | | | | |
|---|---|---|---|---|---|
| | – n (%) | + n (%) | ++ n (%) | +++ n (%) | Chi-square P value |
| All ($n=89$) | 12 (13) | 23 (26) | 29 (33) | 25 (28) | |
| Nuclear grade 1 ($n=2$) | 0 | 1 (50) | 1 (50) | 0 | |
| Nuclear grade 2 ($n=29$) | 8 (28) | 10 (34) | 5 (17) | 6 (21) | |
| Nuclear grade 3 ($n=58$) | 4 (7) | 12 (21) | 23 (40) | 19 (33) | 0.04 |
| Comedo ($n=47$) | 1 (2) | 12 (26) | 15 (32) | 19 (40) | |
| Non-comedo ($n=42$) | 11 (26) | 11 (26) | 14 (34) | 6 (14) | 0.002 |
| No microinvasion ($n=71$) | 12 (17) | 21 (30) | 21 (30) | 17 (24) | |
| Microinvasion ($n=18$) | 0 | 2 (11) | 8 (44) | 8 (44) | 0.04 |
| ER- ($n=20$) | 1 (5) | 2 (10) | 9 (45) | 8 (40) | |
| ER+ ($n=44$) | 8 (18) | 12 (27) | 14 (32) | 10 (23) | 0.13 |
| PR- ($n=32$) | 1 (3) | 5 (16) | 12 (37) | 14 (44) | |
| PR+ ($n=31$) | 8 (26) | 8 (26) | 11 (35) | 4 (13) | 0.008 |
| Ki-67+ ($n=43$) | 2 (5) | 9 (21) | 15 (35) | 17 (39) | |
| Ki-67- ($n=18$) | 5 (28) | 3 (17) | 7 (39) | 3 (17) | 0.04 |

ER, Oestrogen receptor; PR, Progesterone receptor.

known prognosis was chosen. Comedo or cribriform tumours were more frequently Tn-C-positive than other types (Table 1). Cribriform tumours were often positive for ER (8/9) and PR (6/9), and they expressed Tn-C periductally in 10/12 tumours (in 9/12 moderate to strong staining) and stromally in 6/12 tumours (Table 1).

The age of the patient was not related to Tn-C expression nor was the size of the tumour (data not shown). The tumours with a known size and the mainly multifocal tumours with no size definition were also compared and no difference was found in Tn-C distribution (data not shown).

The DCIS tumours that expressed (moderate to strong) periductal Tn-C were associated with microinvasion ($P=0.04$), comedo type ($P=0.002$), high nuclear grade ($P=0.04$), lack of PR ($P=0.008$) and positive Ki-67 expression ($P=0.04$) (Table 2). They tended to be ER-negative, but this association did not reach statistical significance. Stromal Tn-C expression was associated with periductal Tn-C expression ($P=0.05$) and microinvasion ($P=0.005$). A non-significant association with positive Ki-67-immunoreactivity was also noted ($P=0.07$; Table 3).

Microinvasion was considered to be present if it was mentioned in the original pathology report on paraffin samples or if it was noted in the sections made for this study. There was microinvasion in 18/89 tumours (20%). It was associated with periductal Tn-C expression ($P=0.04$), stromal Tn-C

*Table 3. Association between tenascin-C (Tn-C) expression in the stroma and other variables of intraductal carcinoma (DCIS)*

| | Tn-C positive in stroma n (%) | Chi-square P value |
|---|---|---|
| All ($n=89$) | 26 (29) | – |
| Tn-C periductal- ($n=12$) | 1 (8) | |
| Tn-C periductal+ ($n=23$) | 2 (9) | |
| Tn-C periductal++ ($n=29$) | 8 (28) | |
| Tn-C periductal+++ ($n=25$) | 11 (44) | 0.02 |
| Nuclear grade 1 ($n=2$) | 1 (50) | |
| Nuclear grade 2 ($n=29$) | 6 (21) | |
| Nuclear grade 3 ($n=58$) | 15 (26) | 0.6 |
| Comedo ($n=47$) | 12 (26) | |
| Non-comedo ($n=42$) | 10 (24) | 0.9 |
| Microinvasion ($n=18$) | 9 (50) | |
| No microinvasion ($n=71$) | 13 (18) | 0.005 |
| ER- ($n=20$) | 4 (20) | |
| ER+ ($n=44$) | 13 (30) | 0.4 |
| PR- ($n=32$) | 9 (28) | |
| PR+ ($n=31$) | 8 (26) | 0.8 |
| Ki-67 + ($n=43$) | 15 (35) | |
| Ki-67 - ($n=18$) | 2 (11) | 0.07 |

ER, Oestrogen receptor; PR, Progesterone receptor.

*Table 4. Distribution of periductal and stromal expression of tenascin-C (Tn-C) in purely intraductal carcinomas (DCIS)* (n = 89) *and in the intraductal component of small invasive ductal carcinomas* (n = 44)

| | Pure DCIS (%) | Intraductal component of invasive ductal carcinoma* (%) | *P* value |
|---|---|---|---|
| Tn-C periductal | | | |
| – | 12 (13) | 2 (5) | |
| + | 23 (26) | 6 (14) | |
| ++ | 29 (33) | 20 (45) | |
| +++ | 25 (28) | 16 (36) | 0.1 |
| Tn-C stromal | | | |
| – | 67 (75) | 8 (18) | |
| + | 9 (10) | 11 (25) | |
| ++ | 9 (10) | 13 (30) | |
| +++ | 4 (5) | 12 (27) | < 0.0001 |

*Detailed in [9].

expression ($P = 0.005$), comedo type ($P = 0.02$) and a Ki-67-positive immunoreaction ($P = 0.02$), but not with nuclear grade (data not shown).

*Follow-up of the DCIS patients*

None of the DCIS patients had metastases. The median follow-up for the local status of the patients treated with breast-conserving surgery was 2 years (range 5 months–9 years). Four local recurrences occurred 1.5–5.5 years, median 3.3 years, after the operation. All recurrences were DCIS and no invasive growth was encountered. In 2 patients the resection margin had not been tumour-free and in 1 patient the tumour was multifocal with an uncertain resection margin, the smallest distance from DCIS being 3 mm. 1 patient had not received postoperative radiotherapy and there was no reference to the resection margin in the pathology report. None of the histopathological variables were related to these local recurrences, but because of non-standardised surgical treatment, the series was not suitable for evaluation of local recurrence.

*Tn-C in pure DCIS and in the intraductal component of invasive carcinomas*

There was no significant difference in periductal Tn-C distribution in the pure DCIS group and the 44 small, invasive ductal carcinomas with an intraductal component [9] (Table 4). Stromal Tn-C expression was present in 25% of the pure DCIS and in 82% of the intraductal components ($P < 0.0001$; Table 4).

## DISCUSSION

In this study of 89 DCIS tumours, an increased periductal expression of Tn-C was correlated with microinvasion and with comedo type. Comedo type is known to be associated with a higher risk of both invasion [11] and recurrence [12, 13]. Tn-C was also associated with lack of PR expression and with cell proliferation, measured by the Ki-67 antigen. Using *in situ* hybridisation, it has recently been shown that Tn-C is produced by the carcinoma cells of early invasive nests of DCIS and that these mRNA-positive cells are particularly frequent at the margins of the carcinoma cell nests [4, 5]. This is consistent with our observations of a connection between Tn-C and early invasion.

Tn-C expression in morphogenesis and in carcinogenesis have marked resemblances. Tn-C accumulates specifically in the dense mesenchyme around epithelial organ primordia [22] and in invasive nests of breast carcinoma [5, 6]. The source of Tn-C at these sites is predominantly or entirely the epithelial cells themselves [4, 5, 23]. After differentiation, this Tn-C production is downregulated [22–24]. An analogous downregulation may take place in infiltrating breast carcinomas, where the expression at the invading periphery is often intense, whereas the central parts of the tumour express less Tn-C [4, 6]. This downregulation of Tn-C expression within fully evolved advanced carcinomas may explain why Tn-C-negative tumours have a worse prognosis in breast cancer [25] and colorectal cancer [26]. The origin of the stromal Tn-C in infiltrating carcinomas may be different, produced by fibroblasts and related to desmoplasia, as shown in the scirrhotic type of breast carcinoma [5]. This type of tumour represents 'the wound that does not heal' [1] and Tn-C may reflect and participate in a process resembling wound healing or inflammation related to host-response and different from cancerous invasion. It seems as if carcinoma cells produce Tn-C at the invasion front in a manner similar to actively growing epithelia during fetal development.

The distribution of Tn-C in other benign, premalignant and malignant epithelial tissues resembles that of the breast. In colon adenomas, Tn-C expression in the basal lamina is increased as compared with the normal mucosa and in invasive adenocarcinomas Tn-C is found in the basal lamina and also in the stroma [27]. Analogous changes in expression have been reported in the prostate [28], the endometrium [29], the cervix uteri and the vulva [18], the urinary bladder [30], in salivary gland tumours [31], lung tumours [32], and premalignant oral lesions and squamous cell carcinoma [33]. It seems possible that measurement of Tn-C may be of clinical use and aid the identification of early stages of invasion in any carcinoma. Future studies are needed to show whether Tn-C can be used to help decisions on the extent of surgery and the need for radiotherapy and adjuvant medication in early carcinomas.

*In vitro* Tn-C participates in the control of cell proliferation and migration. According to both *in situ* hybridisation and immunohistochemical studies, Tn-C is produced by breast carcinoma cells at the site of early invasion and may, thus, have an active function in cancer invasion and metastasis. The expression of Tn-C in the invasion border of small infiltrating breast carcinomas predicts both local recurrence after breast-conserving surgery [10] and distant metastasis [9]. In the present study, moderate to strong periductal expression of Tn-C in DCIS was associated with comedo type, microinvasion, high nuclear grade, high Ki-67 expression and lack of PR. Expression in the stroma was associated with moderate to strong periductal staining and microinvasion. Enhanced periductal Tn-C expression may be a sign that DCIS is likely to progress into invasive breast cancer and it may aid in finding microinvasion. The stromal expression of Tn-C was considerably higher in the intraductal component of early infiltrating breast carcinomas than in pure DCIS. We suggest that Tn-C is related to invasion and it may be of value when measuring disease character and selecting treatments for carcinomas.

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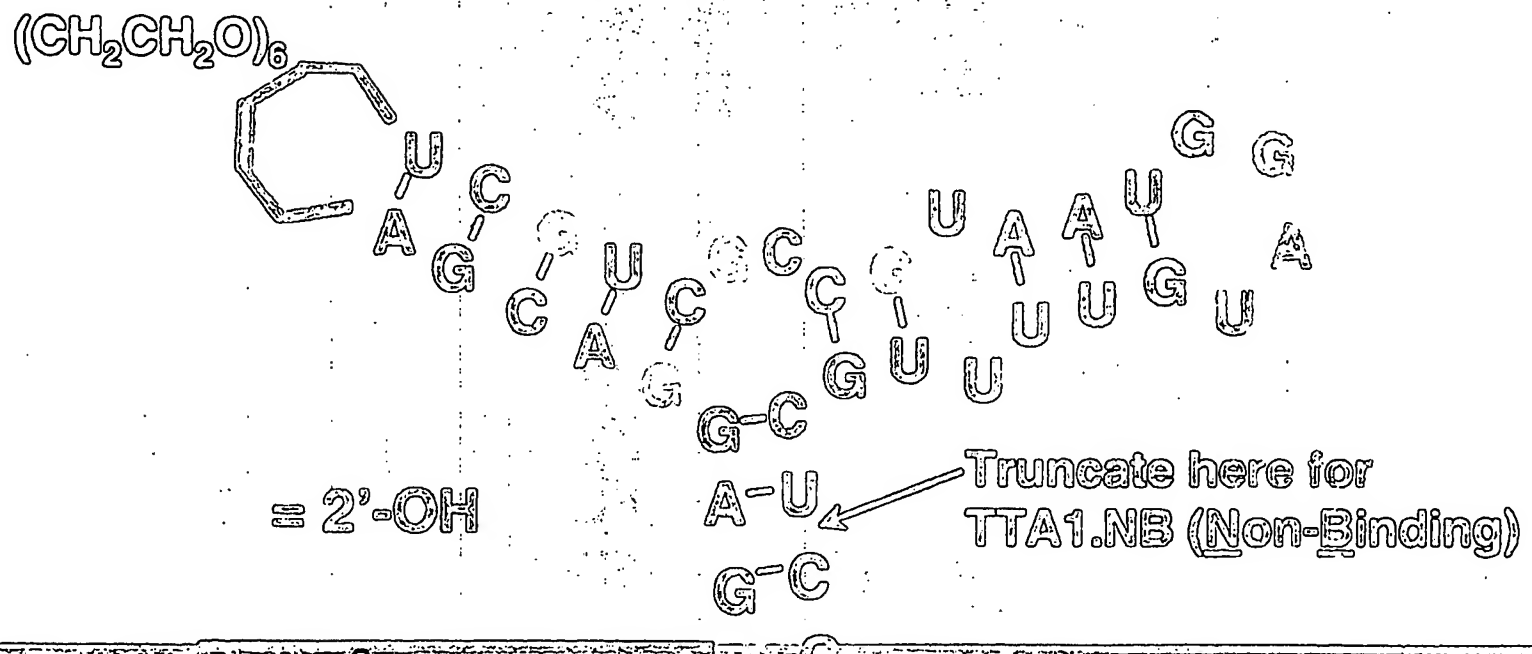
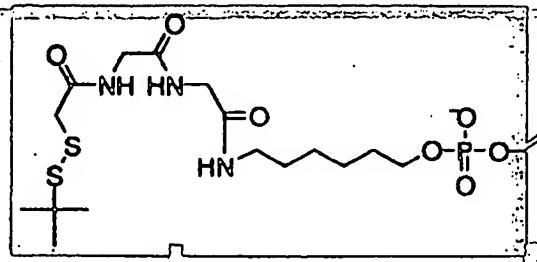
# Tenascin-C Aptamer TTA1

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**Tumor Targeting by an Aptamer.** B.J. Hicke, Y-F Chang, T. Gould, C. Marion, S. Warren, P.G. Schmidt.

Aptamers are oligonucleotides generated from a combinatorial chemistry process called SELEX (Systematic Evolution of Ligands by EXponential enrichment). As small globular nucleic acids (7-20 kDa), aptamers are distinct from anti-sense oligonucleotides. Aptamers typically have high affinity for protein targets (0.01-10 nM).

We have studied the tumor uptake and biodistribution of an aptamer against tenascin-C. The aptamer, TTA1, binds with high affinity (5 nM) and specificity to the target protein. Tenascin-C is an extracellular matrix protein that is over-expressed during formation of a variety of tumor types. These include carcinomas of the breast, lung, prostate and colon; melanoma, and glioblastoma.

Tumor targeting by the aptamer was tested using human U251 gliobastoma tumors xenografts implanted sub-cutaneously in athymic mice. TTA1 was prepared by solid state synthesis with technitium cage linked to the 5' end of the oligonucleotide. The 99mTc-labeled aptamer was administered iv at 3.25 mg/kg. Blood clearance is rapid; t1/2 < 2 min. At 1 hr, tumor uptake is 2.7% ID/gm, while a non-binding control aptamer displays low tumor uptake, 0.15% ID/gm. **TTA1's tumor/blood (T/B) ratio is 25 at 1 hr, 100 at 9.5 hr, and 200 at 17 hr.** After entering the tumor the aptamer remains there, with little decrease in %ID/gm between 3 and 17 hr. By 9.5 hr, tumor uptake is greater than 10-fold higher than all measured organs except intestines, which are equivalent to tumor.

The aptamer radioactivity displays very high tumor/tissue ratios and the signal persists in the tumor xenograft. These features, combined with the chemical versatility of aptamers as compared to antibodies and peptides, suggest that the aptamer may be exploited for imaging and therapeutic applications.

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# Tenascin-C Aptamers Are Generated Using Tumor Cells and Purified Protein*

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**Tenascin-C (TN-C) is an extracellular matrix protein that is overexpressed during tissue remodeling processes, including tumor growth. To identify an aptamer for testing as a tumor-selective ligand, SELEX (systematic evolution of ligands by exponential enrichment) procedures were performed using both TN-C and TN-C-expressing U251 glioblastoma cells. The different selection techniques yielded TN-C aptamers that are related in sequence. In addition, a crossover procedure that switched from tumor cell to purified protein selections was effective in isolating two high-affinity TN-C aptamers. When targeting tumor cells *in vitro*, the observed propensity of naive oligonucleotide pools to evolve TN-C aptamers may be due to the abundance of this protein. *In vivo*, TN-C abundance may also be well suited for aptamer accumulation in the tumor milieu. A size-minimized and nuclease-stabilized aptamer, TTA1, binds to the fibrinogen-like domain of TN-C with an equilibrium dissociation constant ($K_d$) of $5 \times 10^{-9}$ M. At 13 kDa, this aptamer is intermediate in size between peptides and single chain antibody fragments, both of which are superior to antibodies for tumor targeting because of their smaller size. TTA1 defines a new class of ligands that are intended for targeted delivery of radioisotopes or chemical agents to diseased tissues.**

Tenascin-C is a very large (>1 × $10^6$ Da) hexameric glycoprotein that is located primarily in the extracellular matrix (ECM).[1] TN-C is expressed during fetal development, wound healing, tumor growth, atherosclerosis and psoriasis, suggesting a role for this protein in tissue remodeling processes (reviewed in Refs. 1 and 2; see also Refs. 3–5). As judged by Western blotting and immunohistochemical staining (6–16), TN-C levels in tumors are significantly higher than in normal tissue. Further, TN-C levels are predictive of local tumor recurrence and are correlated with invasiveness and distant metastasis (17–19), although these findings remain controversial. Tumor metastases can also express TN-C (10, 20). In addition to localization in tumor stroma, TN-C can be associated with tumor vascular structures (21–24) and may promote angiogenesis through interaction with the integrin $\alpha_v\beta_3$ (25). Because of the abundance of TN-C in tumor stroma and its association with angiogenesis, high-affinity TN-C ligands may be clinically useful tumor-targeting agents. In fact, radiolabeled antibodies to TN-C are currently being evaluated in glioblastoma patients (26, 27) with significant responses to treatment in a phase II study (28).

Aptamers are typified by high affinity and specificity for their cognate proteins (reviewed in Refs. 29–31) and can be considered as oligonucleotide analogs of antibodies. However, as nucleic acids, aptamers are fundamentally distinct from antibodies. In having small size (8–15 kDa) relative to antibodies (150 kDa), aptamers are candidates for rapid tumor penetration and blood clearance. These are useful attributes for noninvasive diagnosis of disease (32) and may provide advantages over antibodies and fragments thereof, which demonstrate slower tissue penetration and clearance rates. To identify an aptamer for investigation of tumor-targeting and blood clearance properties, we describe herein a SELEX process to identify TN-C aptamers and then focus attention on a single aptamer, TTA1.

The SELEX process uses large ($10^{14}$-$10^{15}$ sequences) oligonucleotide pools to identify binding species, *i.e.* aptamers, to a variety of purified molecular targets. In addition to generating aptamers against purified proteins/small molecules, SELEX technology can generate aptamers to cells (33) and tissues.[2] The advantages of complex targets include freedom from the need to define and purify a molecular target, and presentation of proteins in native folding and glycosylation states. For complex SELEX experiments, identifying optimal selection conditions is theoretically possible (34) but remains a challenging task. In contrast, selection against purified protein allows ready experimental manipulation to achieve optimal enrichment of high-affinity aptamers (35) and requires no deconvolution to identify the cognate protein (or lipid, oligosaccharide, nucleic acid, etc.). Relative to cells and tissues, purified proteins often exhibit lower nonspecific binding of nucleic acids, and therefore selections proceed more rapidly. Because each has advantages, we elected to use both purified protein and cells as target sources to obtain TN-C ligands.

A previous SELEX experiment targeting U251 glioblastoma cells identified a DNA aptamer that binds to tenascin-C,[3] demonstrating that TN-C is a selectable target on U251 cells. The ssDNA aptamer displays greatly reduced binding affinity at

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[1] The abbreviations used are: ECM, extracellular matrix; SELEX, systematic evolution of ligands by exponential enrichment; TN-C, tenascin-C; ssDNA, single-stranded DNA; SPR, surface plasmon resonance; RT-PCR, reverse transcription-PCR.

[2] A. Stephens, personal communication.

[3] H. Chen and D. Daniels, personal communication.

physiological temperatures,[4] perhaps because these initial cell SELEX experiments were performed at 4 °C. Thus the aptamer has relatively low affinity ($K_d$ ~100 nM) at 37 °C and, being composed of DNA, is susceptible to nuclease activity *in vivo*. These features render the DNA aptamer unsuitable for *in vivo* applications. To identify aptamers for use as tumor-targeting agents, we undertook SELEX experiments at 37 °C using a nuclease-stabilized 2'-F pyrimidine oligonucleotide library.

## EXPERIMENTAL PROCEDURES

*Cells, Proteins, and Oligonucleotides*—U251 cells, derived from a human glioblastoma, were obtained from the National Cancer Institute-Frederick Cancer Research Facility Tumor Repository and cultured in RPMI 1640 (Life Technologies, Inc.) + 10% fetal bovine serum (Summit, Ft. Collins, CO) to 90% confluency on tissue culture-treated polystyrene. Viability was checked using trypan blue staining. Human TN-C (Chemicon, Temecula, CA; other sources were inferior in purity and activity) was stored as frozen aliquots at −80 °C. After thawing, preparations could be stored for at least 2 months at 4 °C without loss of aptamer binding activity. Purified recombinant tenascin-C fragments (36) were obtained from Harold P. Erickson, Duke University. Human and bovine serum albumin (fraction V) were obtained from Sigma. DNA oligonucleotides were obtained from Operon (Alameda, CA), and aptamer synthesis was performed at NeXstar Pharmaceuticals as described (37). Specialty phosphoramidites were obtained from Glen Research (Sterling, VA; $(CH_2CH_2O)_6$ = Spacer 18; hexylamine; 3'dT polystyrene support), JBL Laboratories (San Luis Obispo, CA; 2'-F C and 2'-F U), or Proligo (Boulder, CO; PAC-protected 2'-$OCH_3$-G, rG, and rA). 5'-amine-containing oligonucleotides were conjugated to succinimidyl biotin (Pierce) in 30% dimethyl formamide, 200 mM sodium borate, pH 9.0, at 25 °C for 15 min and purified on polyacrylamide gels. Alternatively, aptamer transcripts were 5'-biotinylated using a method described elsewhere.[5] In brief, transcription of DNA templates is initiated with a 5-fold excess of a modified GMP over GTP in order to efficiently place a unique biotin on the 5' end of the transcript. The modified GMP bears a hexyl amine moiety on the 5' position that has been conjugated to biotin using the succinimidyl chemistry described above.

*SELEX Procedures*—These procedures were generally performed as described (38). To prepare the initiating random library, double-stranded transcription templates were prepared by Klenow fragment extension of 40N7a ssDNA: 5'-TCGCGCGAGTCGTCTG(40N)CCGCATCGTCCTCCC-3'. The reverse complement of this sequence is the "sense" strand, representing the fixed sequences that span the random regions shown for individuals in Fig. 5. This was done using the 5N7 primer: 5'-TAATACGACTCACTATAGGGAGGACGATGCGG-3', which contains the T7 polymerase promoter (underlined). The $^{32}$P-body labeled library was prepared with T7 RNA polymerase; all transcription reactions were performed in the presence of 2'-F pyrimidine nucleotides and 2'-OH purine nucleotides. For cell selections, U251 cells were grown to 90% confluence on 150-mm-diameter tissue culture plates and washed three times with 10 ml of binding buffer (Dulbecco's phosphate-buffered saline with $MgCl_2$ and $CaCl_2$ (Life Technologies, Inc.) and 0.05% bovine serum albumin). 1500 pmol of $^{32}$P body-labeled library was then incubated with the cells in 10 ml of binding buffer for 45 min with gentle shaking. Unbound oligonucleotides were removed using seven washes (10 min each) of 10-ml binding buffer, typically removing >99% of input radioactivity. A final 20–40-min wash of 5 ml included 10 mM EDTA that caused U251 cells to detach. Cells were pelleted (5 min at 300 × *g*) and the supernatant removed (the EDTA elution). Plates were then treated with 1 ml of Trizol (Life Technologies, Inc.), and the remaining cells/extracellular matrix were removed using a cell scraper. To form the final Trizol elution, pelleted cells from the EDTA elution were added to the Trizol extraction from the plate. After the first round of selection, the EDTA elution pool and Trizol elution pools were kept separate. In the Trizol arm, EDTA-sensitive aptamers were eluted and discarded before Trizol elution. All washes and incubations were at 37 °C in binding buffer. For EDTA elutions, the sample was extracted three times with a 1:1 mixture of phenol/chloroform and once with chloroform. To recover additional radioactivity, organic phases were back-extracted with 100 μl of 10 mM Tris, pH 7.5, and an additional volume of chloroform. Nucleic acid was then precipitated twice using 2 M $NH_4OH$ and 2.5 volumes of ethanol. For the Trizol elution, an additional 0.2 volume of chloroform was added to facilitate phase separation. The aqueous phase was then extracted twice with phenol:chloroform and once with chloroform and then treated with 5–10 μg of RNase A for 10 min at 37 °C to degrade contaminating cellular RNA (the aptamers are resistant to RNase A by virtue of 2'-F-modified pyrimidines). Organic phases were back-extracted as described for the EDTA elution. To precipitate nucleic acids, 0.25 volume of 0.8 M sodium citrate, 1.2 M sodium chloride was added along with 0.25 volume of isopropanol. Reverse transcription (RT)-PCR and transcription were performed as described (38). Two synthetic primers, 5N7 (see above) and 3N7a (5'-TCGCGCGAGTCGTCTG-3'), were used for RT-PCR. To monitor aptamer pool complexity, renaturation rates were measured as described (39).

*Plate SELEX Procedures*—These procedures were performed as described (40). For each round, 96-well Lumino plates (Labsystems, Needham Heights, MA) were coated for 2 h at room temperature with 200 μl of Dulbecco's phosphate-buffered saline containing tenascin-C. Control wells lacked tenascin-C in this initial coating step. After being coated, the wells were blocked using HBSMC buffer (20 mM HEPES, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 g/liter human serum albumin) for rounds 1–5. For rounds 6–8, wells were blocked with HBSMC buffer containing 1 g/liter casein (I-block; Tropix). This switch of blocking agent was performed to decrease background binding of aptamer pools to the plate surface. Binding and wash buffer consisted of HBSMC buffer containing 0.1% Tween 20. The aptamer pool was diluted into 100 μl of binding buffer and incubated for 30 min at 37 °C in the protein-coated wells. After binding, six washes of 200 μl each were performed. The wells were then emptied and placed on top of a 95 °C heat block for 5 min ("heat elution"). Standard avian myeloblastosis virus (AMV)-reverse transcriptase reactions (50 μl) were performed at 48 °C directly in the well, and the reaction products were utilized for standard PCR and transcription reactions. Cloning and sequencing used standard procedures.

*Binding Assays*—To measure aptamer pool binding, U251 cells were grown to confluence in 12-well tissue culture plates (Falcon 3047, Becton Dickinson). After the cells were washed with binding buffer, $^{32}$P body-labeled aptamer pools were incubated with cells in binding buffer for 40 min at 37 °C. Unbound radioactivity was removed by aspiration, and two 10-s washes were performed. Trizol was used to collect bound cpm, which were quantitated by liquid scintillation counting.

For SPR, aptamer pools were 5'-biotinylated (described above) and immobilized to a streptavidin-containing surface (SA chip, BIACORE 2000, Biacore AB, Uppsala, Sweden) at a level of ~1000 response units. Running buffer was HBSMC containing 0.005% Tween 20 (P20, Biacore AB). A reference flow cell for each experiment consisted of a random sequence oligonucleotide pool. Kinetic constants for TN-9 binding to TNfbg (the bacterially expressed fibrinogen-like domain) were determined using standard methods (41, 42). Nitrocellulose filter partitioning assays were performed as described (43). Briefly, $^{32}$P end-labeled oligonucleotides at 0.5 × $10^{-10}$ M were incubated with increasing concentrations of TN-C in HBSMC buffer + 0.01% (w/v) human serum albumin at 37 °C for 15 min. Reactions were then filtered over nitrocellulose, and bound cpm were quantitated. Data were fit to obtain binding constants as described (44).

To measure binding of aptamers to protein immobilized on plates, anti-tenascin-C monoclonal antibodies (mTN12, mouse TN-specific, Sigma; HxBO6, human TN-specific, Harold Erickson, Duke University) were adsorbed to MicroLite-2 96-well plates (Dynex Technologies) in HBSMC buffer for 18 h at 4 °C. Wells were washed four times with HBSMC buffer and blocked using 200 μl of HBSMC buffer + 0.1% (v/v) I-block (Tropix) for 2 h at 22 °C. Tenascin-C was then captured by incubation with serum-free medium from cells expressing mouse TN-C (3t12, ATCC) or human TN-C (U251, described above) for 18 h at 4 °C. After six washes with 200 μl of HBSMC buffer containing 0.1% I-block and 0.05% Tween 20 (HBSMCIT buffer), 150 μl of 5'-biotinylated aptamer was incubated with each well for 30 min at 37 °C. This was followed by three washes of 10 s each (aptamers tend to have rapid on and off rates relative to antibodies) with HBSMCIT buffer, incubation with a 1:1000 dilution of streptavidin-alkaline phosphatase (Roche Molecular Biochemicals), and 3–5 washes in HBSMCIT. Bound aptamer/streptavidin-AP complexes were quantified by chemiluminescent detection using CSPD/Sapphire (Tropix/ABI) according to the manufacturer's instructions. Briefly, 700 μl of disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3,7}$]decan]-4-yl)phenyl phosphate, 1.2 ml of Sapphire were added to 10 ml of diethanolamine buffer, and 150 μl of the solution was added to each well. After 15 min at 22 °C in the dark, chemiluminescence was detected using a luminometer (LB 96P, Berthold, Nashua, NH).

[4] B. Hicke, data not shown.

[5] United States Patent No. 60/034,651 filed January 8, 1997.

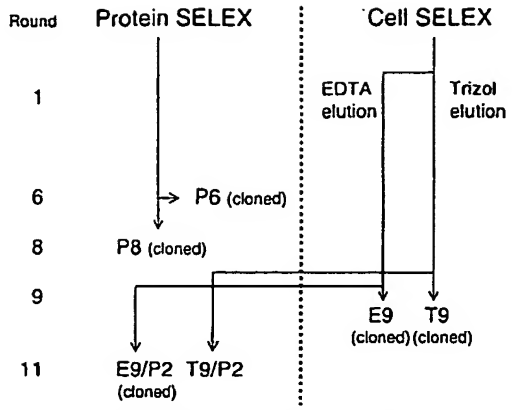


FIG. 1. **Tripartite SELEX experiment for TN-C aptamers.** A random sequence 2'-F pyrimidine RNA library was incubated with either purified tenascin-C or tenascin-C-expressing U251 cells. The iterative SELEX procedure was carried out to identify aptamers. Protein and two-tumor cell SELEX procedures were kept separate, as defined by the *dotted line*. The protein SELEX procedure was carried out by hydrophobic adsorption of TN-C to polystyrene 96-well plates, whereas the cell-based procedure used adherent U251 glioblastoma cells. Two methods of eluting bound aptamers from cells were used, EDTA and Trizol. A crossover SELEX experiment was also performed, symbolized by the *horizontal lines* crossing from *Cell SELEX* to *Protein SELEX*. Here, the ninth round cell SELEX aptamer pools were selected for two rounds against purified tenascin-C. The total numbers of rounds performed are indicated on the *left*.

## RESULTS

To identify aptamers to TN-C, a tripartite SELEX experiment was carried out as diagrammed in Fig. 1. In the first arm, purified TN-C was used. The second arm consisted of selection against a TN-C-expressing glioblastoma cell line, U251. This arm was subdivided into EDTA and Trizol "elutions" to recover bound aptamers. A third arm was a crossover from cell selections onto purified protein selections.

*A SELEX Experiment Using Purified TN-C*—Human TN-C was adsorbed to polystyrene 96-well microtiter plates. To initiate selections, a random aptamer pool consisting of $10^{14}$ oligonucleotides was generated using RNA polymerase. The oligonucleotides contained 2'-F pyrimidines and 2'-OH purines with a 40-nucleotide random sequence region flanked by fixed sequences for RT-PCR. Selections were performed according to Drolet *et al.* (40), essentially consisting of protein-oligonucleotide incubations, washes to remove unbound oligonucleotides, and RT-PCR amplification of the bound oligonucleotides. The amounts of protein with each well in a 96-well plate and the amount of input RNA are indicated in Table I.

A qualitative assessment of PCR amplification indicated that background binding of the RNA pools to polystyrene without associated tenascin-C ("no protein" control) was increasing through the initial five rounds. At round 6, the blocking agent was switched from human serum albumin to casein, which resulted in dramatically decreased aptamer pool binding to the no protein control wells. Progress was quantitated by measuring the affinity of $^{32}$P-labeled aptamer pools for TN-C using a nitrocellulose filter capture assay (45). After five rounds, a slight improvement in binding was evident. Coincident with the switch in blocking agent, the amount of TN-C binding in the aptamer pool rose dramatically in round 6. By round 8, affinity had increased at least 1000-fold to an equilibrium dissociation constant ($K_d$) of $3 \times 10^{-9}$ M. As no further affinity improvement was evident in the subsequent round, selection was deemed complete at round 8.

*A SELEX Experiment Using Tumor Cells*—A second experiment used human U251 glioblastoma cells as the target source.

TABLE I
*Tenascin-C, tumor cell, and crossover SELEX procedures: RNA and protein input*

For the purified protein selections, protein input into each well represents the quantity of protein incubated with each well for adsorption, which was then incubated in buffer with the indicated quantity of RNA. Decreases in protein and RNA input occurred as the pool affinities improved. For the tumor cell SELEX experiment, U251 glioblastoma cells were grown to confluence in tissue culture plates for each round.

| Tenascin-C SELEX experiment | | |
|---|---|---|
| Round | pmol protein/well | pmol RNA/well |
| 1 | 12 (6 wells) | 200 (6 wells) |
| 2 | 12 | 200 |
| 3 | 12 | 200 |
| 4 | 12 | 200 |
| 5 | 2 | 33 |
| 6 | 2 | 33 |
| 7 | 2 | 33 |
| 8 | 0.2 | 3.3 |

| Tumor cell SELEX experiment | | |
|---|---|---|
| Round | Plate diameter | pmol RNA/plate |
| | *mm* | |
| E1/T1 | 2 × 150 | 1500 |
| E2/T2 | 150 | 1500 |
| E3/T3 | 150 | 1500 |
| E4/T4 | 150 | 1500 |
| E5/T5 | 150 | 1500 |
| E6/T6 | 150 | 1500 |
| E7/T7 | 150 | 1500 |
| E8/T8 | 150 | 1500 |
| E9/T9 | 150 | 1500 |

| Crossover SELEX experiment: cells to Tenascin-C | | |
|---|---|---|
| Round | pmol protein/well | pmol RNA/well |
| E9P1/T9P1 | 2 | 33 |
| E9P2/T9P2 | 2 | 33 |

These cells construct an ECM containing abundant TN-C (46). Cells were grown to confluence and incubated with $10^{14}$ sequences of a random oligonucleotide pool (identical to that described above) at 37 °C for 1 h. After extensive washing, a final wash buffer containing 10 mM EDTA was applied to elute EDTA-sensitive aptamers. Because nucleic acid structures and nucleic acid-protein interactions often utilize divalent cations, it was expected that EDTA would elute a subset of cell-bound aptamers. The cells were solubilized, nucleic acids were extracted using Trizol™, a reagent that combines chaotropic denaturation of proteins with organic extraction of nucleic acids, and then the remaining aptamers were collected. Thus the EDTA served to elute a subset of bound aptamers, and the subsequent Trizol elution collected all remaining aptamers along with cellular RNAs. Aptamers from both EDTA and Trizol elutions were amplified by RT-PCR and transcribed, closing the first round of this SELEX experiment. Unlike the purified protein SELEX experiment, cell and input RNA concentrations remained constant throughout nine rounds of selection (Table I).

The progress of the cell selections was monitored by measuring the binding of radiolabeled aptamer pools to U251 cells. To analyze the EDTA elution SELEX experiment, Fig. 2*A* compares binding of a control aptamer pool to rounds 3, 5, and 9. The control aptamer pool bound the cells detectably, and binding was saturable. Relative to this nonspecific binding, rounds 3, 5, and 9 showed progressively increasing binding.

Similar to the EDTA elution pools, the Trizol pools showed increased binding compared with a random aptamer pool (Fig. 2*B*). The T9 (Trizol round 9) pool showed less apparent binding than the T5 pool. This was due to increased binding to the polystyrene surface (data not shown). This outcome suggests

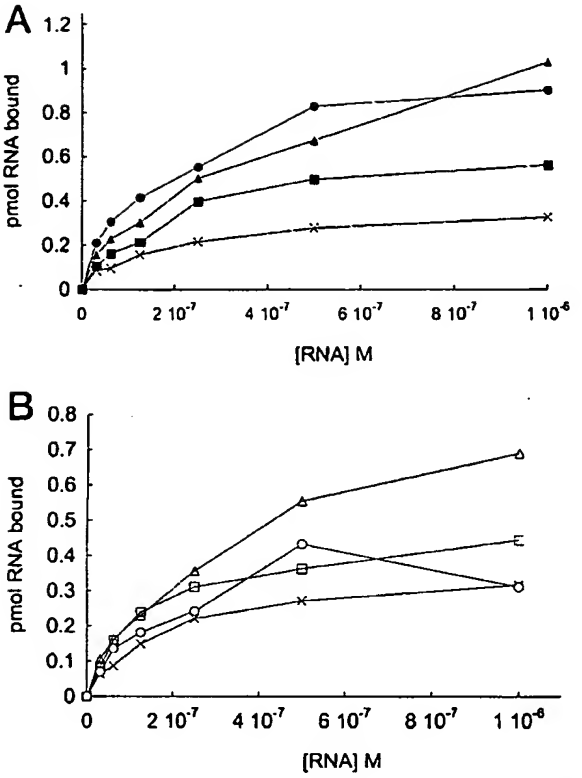


FIG. 2. **Binding of aptamer pools to tumor cells: progress of the SELEX experiment.** U251 tumor cells were grown to confluence. EDTA-eluted (*A*) or Trizol-eluted (*B*) aptamer pools of rounds 3, 5, and 9 from the cell SELEX procedure were labeled with $^{32}P$ and incubated at increasing concentrations with the U251 cell monolayers followed by brief washing. Bound oligonucleotide was quantitated by solubilizing cells with Trizol and scintillation counting. *X*, random aptamer pool; *filled squares*, EDTA round 3 (E3) pool; *filled triangles*, E5 pool; *filled circles*, E9 pool; *open squares*, Trizol round 3 (T3) pool; *open triangles*, T5 pool; *open circles*, T9 pool. The background binding value for a tissue culture well without cells is subtracted for each data point.

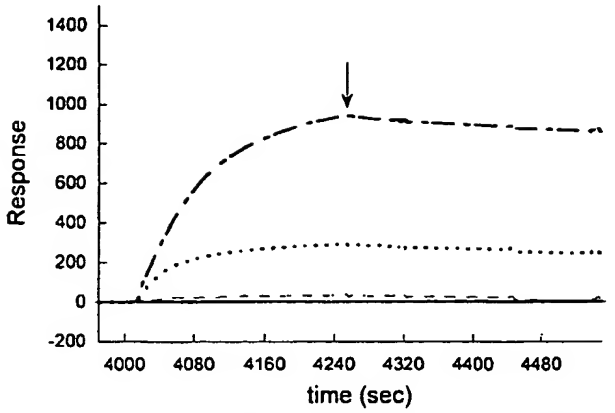


FIG. 3. **Tenascin-C binding to protein- and tumor cell-selected aptamer pools: surface plasmon resonance.** 5'-Biotinylated aptamer pools were immobilized to a streptavidin-bearing carboxylmethyldextran surface in a flow cell (BIACORE 2000). Association with tenascin-C was detected via surface plasmon resonance during injection of $200 \times 10^{-9}$ M protein in buffer at 20 μl/min, and dissociation was observed during buffer flow only after 4250 s (*arrowhead*). Values for binding to a random aptamer pool (200 response units maximum) were subtracted from each data set. ——, random aptamer pool; – – –, Trizol round 9 (T9) pool; ····, EDTA Round 9 (E9) pool; – — – — – —, Protein Round 8 (P8) pool.

that Trizol-eluted aptamers bound to the polystyrene surface, directing selective pressure away from cell binding and toward polystyrene binding.

The cell binding analysis demonstrated pool evolution toward U251 binding. However, this analysis did not fully evaluate the progression of the tumor cell SELEX experiment; this is because a pool of low-affinity ligands for an abundant protein would show higher cell binding than a pool of high-affinity ligands for a rare protein. For many applications, the latter pool is desirable. Therefore we employed another measure of progression, aptamer pool complexity, which can be estimated by measuring nucleic acid renaturation rates ($C_ot$ analysis) (39). Decreasing pool complexity serves as a proxy for convergence upon a high-affinity solution. The $C_ot$ analysis predicted that the E9 pool would contain ~100 different oligonucleotide sequences, whereas the T9 pool would contained ~50,000 sequences (data not shown). Taken together, the cell binding and $C_ot$ analyses indicated that EDTA was more effective than Trizol in driving pool convergence toward cell binding.

To determine whether the U251 aptamer pools contain TN-C aptamers, binding was investigated using a surface plasmon resonance (SPR) assay. Aptamer pools were biotinylated at the 5' terminus and immobilized, via streptavidin, onto the surface of a biosensor chip. TN-C binding was then measured by SPR. Specific binding of TN-C to aptamer pools P8 and E9 was evident, with P8 showing significantly higher binding (Fig. 3). A faint signal was detected in the T9 pool; a low signal was also detected using the filter binding assay described below. Quantitative measurements were not possible because of the large mass of TN-C (> 1 mDa), which slows diffusion through the surface matrix. A further hindrance to quantitation is the hexameric structure of TN-C, which likely causes slow dissociation from the surface because of multivalent interactions. In addition, it was not possible to couple active TN-C to the matrix. The SPR assay using soluble TN-C therefore served a qualitative role; we observed low binding to the tumor cell Trizol arm, increased binding to the tumor cell EDTA arm, and the highest binding to the purified protein arm.

*A Crossover SELEX Experiment Using Tumor Cells and Purified TN-C*—To enrich TN-C aptamer representation in the tumor cell aptamer pools, a crossover SELEX experiment was performed as diagrammed in Fig. 1. The two cell-selected pools were subjected to two rounds of protein selection (Table I), generating pools E9P2 (E9P2 = 9 rounds of EDTA elution from cells and 2 rounds of protein selection) and T9P2. Affinities for TN-C were then determined using a nitrocellulose filter binding assay (Fig. 4). For comparison, the P8 pool was included. This analysis indicated that two rounds of crossover selection on TN-C improved the affinity of E9 by 50-fold. For the Trizol arm, affinity rose from undetectable to $2 \times 10^{-9}$ M in two rounds. Remarkably, just two rounds of crossover selection were required to enrich the high-affinity aptamers that were rare in each tumor cell aptamer pool.

*Isolation and Sequencing of High-affinity TN-C Ligands*—To analyze the content of selected pools, five aptamer pools were cloned and sequenced. Aptamers could be grouped into the three families shown in Fig. 5. Family I members were found in the P8, E9, and T9 pools. These sequences are related through the consensus sequence GACNYUUCN$_{1-3}$GCAYC and have affinities for TN-C ranging from 20 to $100 \times 10^{-9}$ M. The T9 pool contains many different family I sequences, consistent with the high sequence complexity predicted by $C_ot$ analysis (data not shown). Family II members are related through the consensus sequence CGU(C)GCC(G)A. Consistent with their overrepresentation in the P8 and E9P2 pools, family II aptamers have the highest affinities for TN-C. Although family II aptamers from the crossover SELEX procedure (E9P2 clones) are highly related to family II aptamers obtained using purified

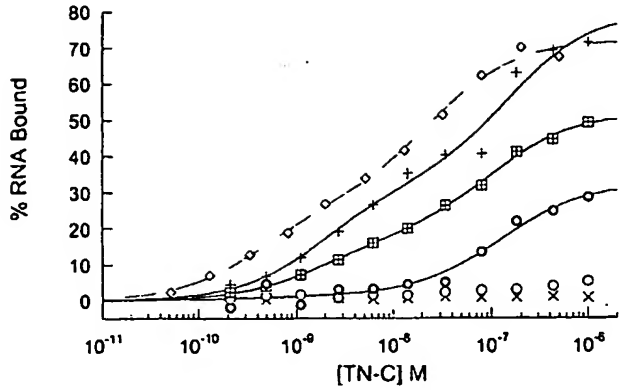


FIG. 4. **Binding of aptamer pools to Tenascin-C: filter partitioning assay.** Aptamer pool binding was measured using a binding assay (45) in which protein-bound $^{32}$P-RNA are partitioned from unbound $^{32}$P-RNA by filtration through nitrocellulose. Tenascin-C binding of cell-selected pools (*X*, random aptamer pool; *open circles*, Trizol round 9 (T9); *filled circles*, E9) was compared with tenascin-C binding after two rounds of crossover selections onto purified protein (*squares with cross*, Trizol R9/Protein R2 (T9P2); *cross*, E9P2) or eight rounds of purified protein selections (*diamond*, P8). The E9/P2, T9/P2, and P8 data sets were fit using a biphasic binding equation, and the E9 pool was fit using a monophasic binding equation (44). Pool $K_d$s reported in Table II refer to the high-affinity component of these binding fits.

protein (TN clones), they arose from distinct sequence lineages. This indicates that cell-derived aptamers did not arise from cross-contamination during the selections. Family III sequences are less common but can also have high affinity for TN-C. Thus, two distinct target sources, cells and protein, identified TN-C ligands that are sequence-related.

*Trends in Selection for High Affinity*—When aptamer pool affinities and family representations were tabulated, trends in TN-C aptamer selection became evident. For example, lower affinity pools (P6, E9, T9) contain more family I members than family II members and vice versa for the higher affinity pools (P8 and E9P2, Table II). This trend is observed for both cell and protein SELEX experiments. Family I and unrelated sequences ("Others") predominate in early, low-affinity populations, whereas family II aptamers emerge later and have higher affinity for TN-C. As discussed elsewhere (34, 35), these trends in selection could be due to higher prevalence of family I aptamers in the random sequence starting pool.

Returning to individual clones, a single family I clone, E9-3, dominates the E9 pool at 24% of the sequences identified (Fig. 5). This overrepresentation indicates that E9 pool had converged upon a small number of sequences. A family II aptamer, TN-9, represented 22% of the P8 pool, whereas family II aptamers E9P2-1 and E9P2-2 together represented 56% of the E9P2 pool. Thus, family II aptamers dominated the advanced pools and displayed high affinity for TN-C with $K_d$s of $1–10 \times 10^{-9}$ M.

*Two Aptamer Epitopes on TN-C*—TN-C is a multidomain protein. It contains epidermal growth factor-like repeats, fibronectin type III repeats, and a fibrinogen-related globular domain at the C terminus (47). To determine the dominant epitope, if any, for the aptamer pools, bacterially expressed TN-C domains (36) were tested for binding. Transcribed aptamer pools were 5′-biotinylated and immobilized onto a streptavidin surface. Binding of TN-C and recombinant domains was then detected by SPR (Fig. 6). An existing ssDNA aptamer, GB41,[3] bound to full-length TN-C as did pool P8 (Fig. 6*A*). Slow dissociation from the surface was observed, most likely because of the large mass and multivalency of TN-C. We then examined three different fragments of TN-C (36) for aptamer and aptamer pool binding. TNfnA–D comprises a series of fibronectin type III repeats; no binding was observed to TNfnA-D (Fig. 6*B*). TNfn3–5, a different series of fibronectin repeats, bound to ssDNA aptamer GB41 but not to the P8 pool we describe here. TNfn3–5 dissociates very rapidly from GB41 at 37 °C (Fig. 6*C*), indicating poor binding at physiologic temperature. In contrast to the ssDNA aptamer, the P8 pool bound to the C-terminal fibrinogen-like domain, TNfbg (Fig. 6*D*). Individual aptamers from families I, II, and III each bound to immobilized TNfbg in the SPR assay (data not shown). Association and dissociation rate constants of aptamer TN-9 for TNfbg were $10^5$ M$^{-1}$ s$^{-1}$ and $10^{-3}$ s$^{-1}$, respectively. However, the $k_{on}$ and $k_{off}$ are considered estimations, as the SPR experiments were not designed to ensure rigorous measure of these rate constants. The $K_d$ derived from these estimates, at $10 \times 10^{-9}$ M, is 10-fold greater than the $K_d$ that was measured using a filter binding assay. In summary, the bacterially expressed domains permit identification of aptamer binding regions on TN-C. To date there are two aptamer epitopes, one within the type III fibronectin repeats and one within the C-terminal fibrinogen-like globule.

*Aptamer Binding Specificity and Species Cross-reactivity*—We next examined the cross-reactivity of a human TN-C aptamer toward mouse TN-C. Because mouse TN-C was not available in purified form, a modified enzyme-linked immunosorbent assay was developed in which TN-C was captured onto a surface and incubated with an aptamer. First, anti-tenascin antibodies were immobilized onto polystyrene 96-well microtiter plates. The wells were then incubated with the tissue culture supernatant of mouse (3t12) or human (U251) cell lines, capturing TN-C onto the surface. Capture was confirmed using a second monoclonal antibody. Aptamer TTA1 (described below), a truncated version of aptamer TN-9, was chosen to test species cross-reactivity. TTA1 was 5′-biotinylated and incubated with captured TN-C at increasing concentrations. After rapid washing, bound TTA1 was quantified. Fig. 7 indicates that half-maximal binding to human TN-C occurred at $\sim 20 \times 10^{-9}$ M, similar to the measured $K_d$ of $5 \times 10^{-9}$ M. In contrast, half-maximal binding to mouse TN-C occurred at $\sim 400 \times 10^{-9}$ M, a 20-fold reduction relative to the human protein. Binding of the control aptamer, TTA1.NB, to the captured TN-C was significantly decreased relative to the binding of TTA1 (Fig. 7). These data indicate that, despite 93% sequence identity between the human and mouse TNfbg domains, aptamer TTA1 has high specificity for human TN-C. Further tests of specificity were conducted by incubating the biotinylated aptamer with adherent cells that express either human or mouse TN-C. We again observed greatly diminished binding of TTA1 to cells expressing mouse TN-C (data not shown). In this case, the cells have developed an ECM, indicating the specificity of TTA1 for a single protein within the native ECM. In general, aptamers selected for binding to a particular protein exhibit low cross-reactivity toward unrelated proteins and even have low cross-reactivity toward highly related proteins. For example, a P-selectin aptamer displays 10,000–100,000-fold selectivity for P-*versus* L- and E-selectin (48).

A final measure of specificity indicates that TTA1 does not bind appreciably to a wide range of extracellular matrix proteins. The radiolabeled aptamer binds to tumor tissue that expresses TN-C but does not bind to tissue that lacks TN-C or to tumors that do not express human TN-C.[6]

*Aptamer TTA1: Size Minimization and Further Nuclease Stabilization*—To prepare an aptamer for *in vivo* experimentation, we made three alterations: size minimization; further stabilization against nuclease activity; and incorporation of a bioconjugation handle for addition of biotin, fluorescent tags,

[6] B. Hicke, unpublished observations.

| Aptamer | SELEX target | Rnd | % of pool | 5′-sequence of variable region-3′ | $K_d$ for TN-C (nM) |
|---|---|---|---|---|---|
| **Family I** | | | | | |
| TN-4 | Protein | P8 | 4 | CAACCUCGAAA GACUUUUCCC GCAUCACUGUGUACUCCCC | 45 |
| TN-32 | | P8 | 4 | CAACCUCAAUCUU GACAUUUCCC GCACCUAAAUUUGCCCC | 15 |
| TN-29 | | P8 | 4 | CCAACCUCAUUUU GACACUUCGCGCACCUAAUUG CCCC | 25 |
| TN-14 | | P8 | <2 | CAAACGAUCACU UACCUUUCCU GCAUCUGCUAGC CUCCCC | 20 |
| E9-3 | Cells | E9 | 24 | ACUAGACCGCGAGUC CAUUCAACUUG CCCAAAAACCUCCCC | 100 |
| Consensus: | | | | CAACC GACNYUUC GCAYC CCCC | |
| **Family II** | | | | | |
| E9P2-1 | Cells/Protein | E9P2 | 21 | UGCCCAUAGAAGCGU GCCGCUAAUGCUAACGCC CUCCCC | 8 |
| E9P2-2 | | | 35 | UGCCCAC UAUGCGU GCCGAAAA ACAUUUCCCCCUCUACCC | 4 |
| TN-7 | Protein | P8 | 7 | AACACUUUCCCAU GCGUCGCC AUACC GGAUAUAUUGCUCC | 7 |
| TN-21 | | | 9 | ACUGGACCAAACCGUCGCCGAUACCCGGAUACUUUGCUCC | 10 |
| TN-9 | | P8 | 22 | AACAAUGCACUCGUCGCCGUAAU GGAUGUUUUGCUCCCUG | 2 |
| Consensus: | | | | CNAY CGU GCCGA RGAU CUCC | |
| **Family III** | | | | | |
| TN-27 | Protein | P8 | <2 | AAACCAACCGUUGACCACCUUUUCGUUUCCGAAAGUCCC | 110 |
| TN-44 | | P8 | 7 | ACGCCAGCCAUUGACCC UCGCUUCCACUAUUCCAUCCCCC | 10 |
| TN-39 | | | <2 | AAGCCAACCCUCUAGUCAGCC UUUCGUUUCCCACGCCACC | ND |
| TN-24 | | | 13 | GACCAACUAAACUGUUCGAAAGCUGGAACAUGUCCUGACGC | 18 |
| Consensus: | | | | GACC UCGNUUCC | |

FIG. 5. **Sequences and affinities of YN-C aptamers.** Aptamers from three SELEX procedures (protein, cell, cell/protein crossover) were grouped into families based on sequence similarity. For simplicity, the fixed sequences at the 5′ and 3′ ends have been omitted. Affinities for TN-C were measured by a nitrocellulose filter binding assay. *% of pool*, aptamer representation in the pool from which it was cloned; *P8*, purified protein SELEX procedure eighth round; *E9*, U251 A EDTA elution ninth round; *T9*, Trizol elution ninth round.

TABLE II

*Family frequencies in aptamer pools*

Five sequence sets are tabulated: selection using protein (P6 = protein sixth round, etc; P8), U251 EDTA elution (E9) and Trizol elution (T9) arms, and cells/protein crossover (E9/P2; E9 + 2 rounds protein). By visual inspection, sequences were grouped into one of four families or were considered as unrelated ("Others"). Elution, method used for eluting bound aptamer during the SELEX protocol. "$K_d$ for TN-C" refers to the affinity of the aptamer pool.

| Target | Elution | Round | $K_d$ for TN-C | Frequency of a sequence family | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | I | II | III | IV | Others |
| | | | | % | | | | |
| Protein | Heat | P6 | 100 nM | 33 | 17 | | | 50 |
| | Heat | P8 | 3 nM | 26 | 40 | 18 | | 16 |
| Cells | EDTA | E9 | 104 nM | 73 | | | | 27 |
| | Trizol | T9 | >1 μM | 15 | | 4 | 11 | 52 |
| Cells/protein | EDTA/heat | E9/P2 | 2 nM | | 64 | | | 36 |

radiometal chelators, etc. Ideally, such changes maintain the affinity and specificity of the aptamer.

A cloned aptamer sequence is typically 70–80 nucleotides in length. For efficient chemical synthesis, it is desirable to identify the minimal high-affinity aptamer sequence. This can be initiated by determining the maximum permissible truncations at the 5′ and 3′ termini. Using described techniques (49) on aptamer TN-9, we found that no nucleotides could be removed from the 5′ terminus, and 16 nucleotides could be removed from the 3′ terminus. This exercise produced aptamer TN-9.4, which has $K_d = 2 \times 10^{-9}$ M for TN-C (Fig. 8).

To identify extraneous nucleotides that reside within the 3′ and 5′ termini required for high-affinity binding to TN-C (*e.g.* internal loops that do not contribute to the protein-oligonucleotide binding interaction), an RNA secondary structure prediction algorithm (50, 51)[7] was utilized. TN-9.4 and its analogous family II sequences, TN-7.4 and TN-21.4, were each subjected to the algorithm. A predicted structure common to all three aptamers was a three-way junction that places the conserved CGUCGCC element at the center of the junction. Of the predicted stems, the distal portion of the second stem did not appear conserved in sequence or length, suggesting that it is dispensable for high affinity binding to TN-C. By chemically synthesizing a series of deletions in this stem, we found that 17 nucleotides could be replaced by a non-nucleotide spacer, $(CH_2CH_2O)_6$, with no affinity loss (TN-9.6, Fig. 8). Although consistent with the three-way junction predicted by the algorithm, this internal deletion does not rule out other potential structures. Together, the truncation and internal deletion analyses enabled trimming of a 55-nucleotide sequence to 39 nucleotides and a small spacer. Such a size reduction is critical for efficient chemical synthesis of the aptamer. As shown in Fig. 8*B*, the size-minimized species has $K_d = 5 \times 10^{-9}$ M, a 5-fold loss in affinity relative to the full-length aptamer.

Pyrimidine positions, as opposed to purines, are the primary source of nucleolytic instability in plasma. Therefore the pyrimidines are protected from nuclease activity by 2′-F groups incorporated during the SELEX procedure. Further stabilization can be achieved by converting purines to 2′-$OCH_3$ purines (52). This occurs after selections, because the addition of 2′-$OCH_3$-modified purines to the existing SELEX procedure causes inefficient transcription. To identify purines that could be converted to 2′-$OCH_3$ without loss of function, aptamer TN-9.4 was divided into five sectors based on the putative three-stem junction structure (Fig. 9). In general, sectors were chosen so that 2′-$OCH_3$ substitutions occurred on one strand of a putative helix, to reduce any helical distortion caused by the substitutions. All purines in each sector were synthetically

[7] Found on the Web at bioinfo.math.rpi.edu/~zukerm/.

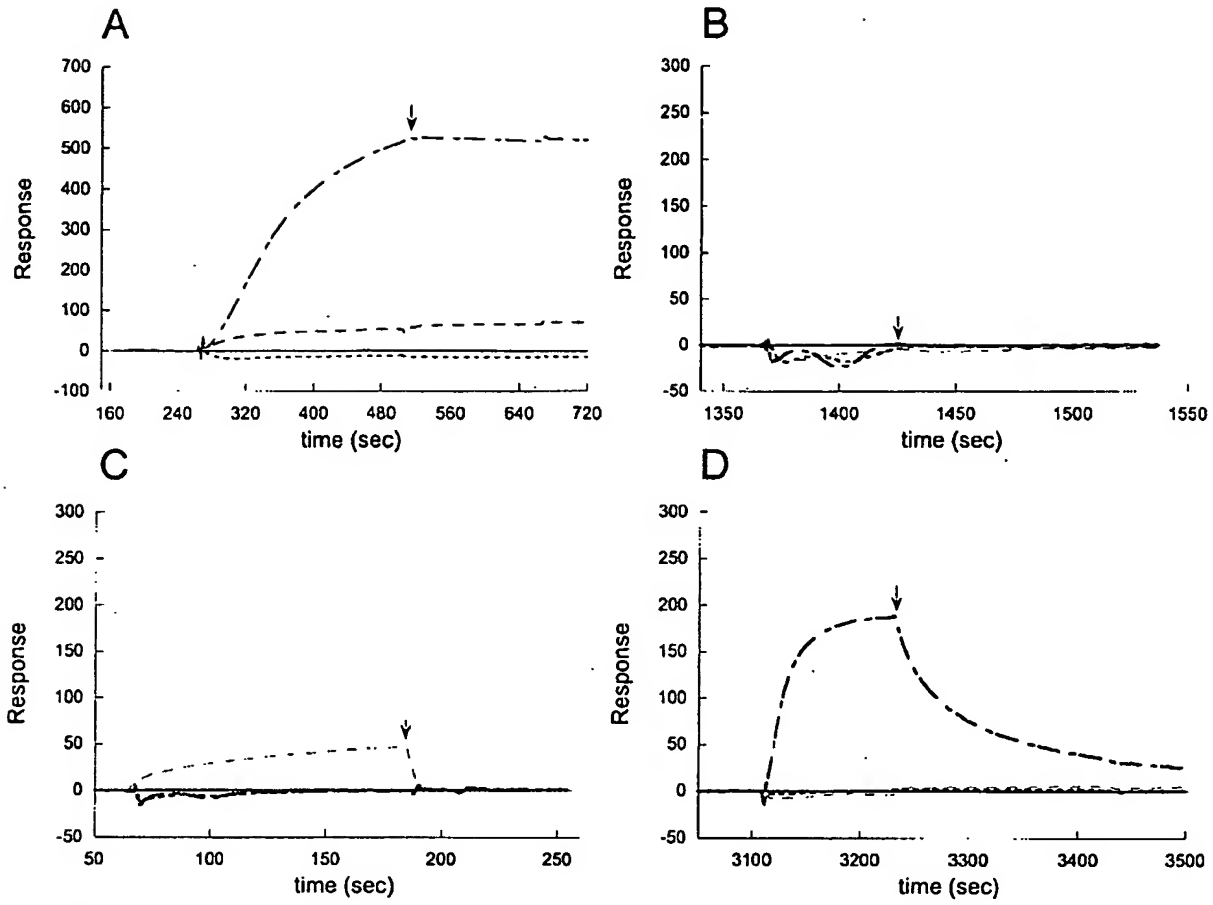


FIG. 6. **SPR assay: binding of tenascin-C fragments to aptamer pools.** 5'-Biotinylated aptamer pools and ssDNA aptamer GB41 were immobilized onto a streptavidin surface. TN-C or bacterially expressed TN-C domains were introduced by flow across the surface at $200 \times 10^{-9}$ M, and binding was measured at 25 °C by SPR (BIACORE 2000). Values for binding to a random aptamer pools were subtracted from each data set. The beginning of the dissociation phase (buffer only) is marked by an *arrowhead*. *A*, binding of full-length tenascin-C. *B*, binding of a tenascin region consisting of fibronectin type III domains A–D (TNfnA–D). *C*, binding of fibronectin type III repeats 3–5 (TNfn3–5). *D*, binding of the fibrinogen-like domain (TNfbg). ——, random aptamer pool; – – –, DNA aptamer GB41; ····, protein round 4 (P4) pool; — — — — — —, P8 pool.

substituted with 2'-$OCH_3$ purines, and affinity for TN-C was measured (Table III). In sectors 2 and 4, binding affinity decreased 20-fold and >1000-fold, respectively, because of the 2'-$OCH_3$ substitutions. We then identified which of the nine purines in sectors 2 and 4 were responsible for affinity loss upon 2'-$OCH_3$ substitution; in the context of complete substitution in sectors 1, 3, and 5, individual purines were substituted and the aptamers tested for affinity (Table III). In so doing, we found that substitution at four of the nine Gs caused loss of affinity: $G^9$, $G^{28}$, $G^{31}$, and $G^{34}$.

Finally, the size minimization and 2'-$OCH_3$-substitution data were combined to synthesize aptamer TTA1 (Fig. 10), a 39-mer. A nonbinding control aptamer, TTA1.NB, was also synthesized. TTA1.NB has a 5-nucleotide deletion; it does not bind TN-C at concentrations up to $1 \times 10^{-6}$ M (data not shown). TTA1 bears a 5' amine for bioconjugations and a 3'–3' cap for exonuclease protection. Pyrimidines contain 2'-F modifications, and purines contain 2'-$OCH_3$ modifications (except the four obligatory 2'-OH Gs and a fifth purine, $G^1$, that also remained 2'-OH). In summary, TTA1 is a nuclease-stabilized 39-nucleotide aptamer that binds human TN-C tightly, with a $K_d$ of $5 \times 10^{-9}$ M.

## DISCUSSION

In this work, our goal was to identify a physiologically active, nuclease-stabilized aptamer that could be tested for tumor targeting capability *in vivo*. A previous tumor cell SELEX experiment yielded a ssDNA aptamer that binds to a major extracellular matrix component, tenascin-C. However, selection at 4 °C resulted in an aptamer that binds moderately well at 4 °C ($K_d \sim 100 \times 10^{-9}$ M)[6] but poorly at physiological temperatures ($K_d > 1 \times 10^{-6}$ M). In addition, DNA is not sufficiently stabilized against nuclease activity to which blood-borne nucleic acids are exposed. Nevertheless, the cell selections had identified a protein that is of significant interest as a marker for tissue remodeling processes including tumor formation. We therefore performed new cell selections and protein selections using a 2'-F pyrimidine library at 37 °C.

In the tumor cell SELEX experiment, EDTA elution was superior to Trizol elution for enrichment of cell-binding aptamers. Because nucleic acid structures and nucleic acid-protein interactions often require divalent cations, we reasoned that low EDTA concentrations could selectively elute a subset of cell- or ECM-bound aptamers. In contrast, the chaotropic agent contained in Trizol removes oligonucleotides indiscriminately from cells, ECM, polystyrene, etc. We found that Trizol elution enriched a population of polystyrene binding sequences that hindered the enrichment of cell-binding aptamers. $C_o t$ analysis predicted far higher sequence complexity in the ninth round Trizol pool than in the ninth round EDTA pool. This prediction was borne out by sequence analysis that showed that the E9

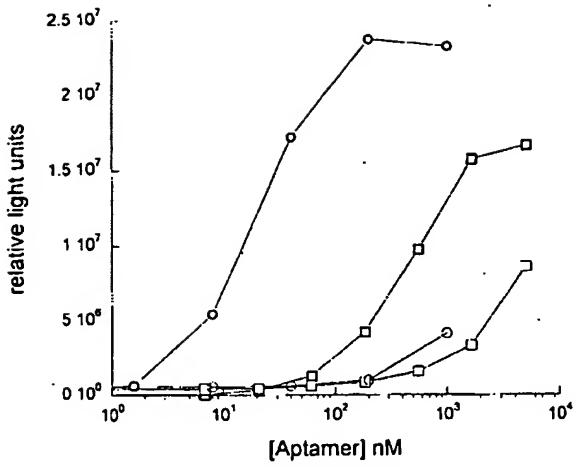


FIG. 7. **Aptamer binding to human and mouse tenascin-C.** Antibodies to human TN-C (HxBO6) and mouse tenascin-C (mTN12) were immobilized to the surface of a 96-well plate and then incubated with medium from tenascin-C expressing human cells (U251 glioblastoma) or mouse cells (3t12 fibroblast). Binding or nonbinding aptamer TTA1 was 5'-biotinylated and incubated with the wells at increasing concentrations. The wells were washed, and bound aptamer was quantified using a streptavidin-alkaline phosphatase conjugate with chemiluminescent detection. Values for the nonbinding aptamer (*open circles*, U251 cell supernatant; *open squares*, 3t12 cell supernatant) were subtracted from values for the binding aptamer, represented as specifically bound aptamer (*filled circles*, U251 cell supernatant; *filled squares*, 3t12 cell supernatant).

pool had repeated sequences, whereas the Trizol pool had no duplicates in 50 analyzed clones. Thus, EDTA elution was superior to Trizol in reducing background binding to polystyrene and in creating an aptamer pool that had converged on a set of cell-binding aptamers.

In the tumor cell SELEX experiment, TN-C was a dominant protein for aptamer selection. There are at least two explanations for this result. First, aptamers bound to membrane targets are candidates for internalization and subsequent degradation by nucleases, and would not be recovered during selections. A second more likely explanation for preferential selection of TN-C aptamers is target abundance. TN-C is present at high (1–10 $\mu$M) concentrations in the ECM (16, 46).[8] In a random oligonucleotide pool, any single aptamer is present at a vanishingly small concentration (~10–20 M). Therefore, concentration of the aptamer target determines the rate and extent of aptamer binding in initial rounds of selection, favoring abundant proteins as targets. The TN-C $\mu$M concentration in the ECM is sufficient to drive the binding of single-copy aptamers in an initial round of the SELEX process and is perhaps sufficient to serve as a dominant target over other less abundant proteins. Furthermore, extended washes were used to remove unbound oligonucleotides. High-affinity aptamers, including the TN-C aptamer described here, typically have $k_{off}$ values of ~ $10^{-3}$ $s^{-1}$ (44, 48, 53), or $t_{1/2}$ values of 2–10 min. After 50 min of washing, ~97% of an aptamer with $t_{1/2}$ = 10 min would be washed away unless the target protein is at sufficiently high concentration for rebinding to occur. Therefore, two factors, extensive washing and the rarity of individual aptamers in a random pool, may each bias cell selections toward abundant proteins. These factors may account for the propensity of two different U251 cell SELEX experiments to isolate tenascin-C aptamers.

For the identification of tumor-targeting aptamers, tumor cells have strengths and weakness relative to purified protein. Using cells enables the presentation of epitopes in their native state and also requires no knowledge of a potential target. The cell SELEX procedure can lead to the identification of new target proteins or the new appreciation of a known protein as an aptamer target. Indeed, this occurred with the initial U251 cell selections that identified an ssDNA aptamer against TN-C, as well as the identification of a new 42-kDa trypanosome protein (54). A disadvantage of cell selections is that slow convergence of cell SELEX pools is caused by higher background binding of nucleic acids to cells (55) than to purified proteins. Furthermore, selection for high affinity may be compromised because concentration of the target protein(s) is unknown. This is illustrated by the experiments in which TN-C ligands dominated the cell selections at round 9, but pool affinity was poor at $K_d$ = 100 × $10^{-9}$ M. This pool was then exposed to purified protein in the crossover procedure. By applying selection pressure for affinity, the $K_d$ for TN-C improved 50-fold in two rounds and isolated two dominant sequences. Therefore high-affinity aptamers were present, but not evident, in the E9 tumor cell pool. By extension, the E9 pool may contain a variety of low abundance/high-affinity aptamers to additional tumor cell proteins of interest. To summarize, cell selections may guide new target identification in unbiased fashion and can be complemented by crossover procedures (or *de novo* SELEX experiments) to select for high-affinity binding to a protein of interest.

The repeated emergence of TN-C as a target suggests that abundant ECM proteins may be generally well suited as targets for cell-based aptamer selections. It is possible that abundant ECM proteins may also be well suited for aptamer-based tissue targeting *in vivo*. However, this hypothesis is currently difficult to test; a key difference between *in vitro* and *in vivo* selection is that after intravenous injection, oligonucleotides must run a gauntlet of nucleases and blood clearance mechanisms before gaining access to a target tissue, decreasing library complexity significantly. Further experiments using *in vivo* aptamer selection are needed to address these issues. A truncated aptamer that can be nuclease-stabilized and modified at will has considerable advantages *in vivo* over the aptamer pools typically used for selections. Therefore aptamers designed for *in vivo* tissue targeting can be readily derived using protein- and cell-based selection techniques, with appropriate post-selection modifications. As tissue- and organism-based SELEX procedures mature, we suggest that abundant ECM proteins may be useful surrogates for identifying tissue-targeting aptamers.

To characterize the binding and epitopes of family I, II, and III tenascin-C aptamers, we used an SPR assay. Each ligand family binds to the C-terminal fibrinogen-like domain, TNfbg. Although experimental conditions were not optimized to determine the kinetics of aptamer binding (41), the $k_{on}$ and $k_{off}$ for aptamer TN-9 were ~$10^5$ $M^{-1}$ $s^{-1}$ and $10^{-3}$ $s^{-1}$, respectively. This corresponds to a $K_d$ of 1 × $10^{-8}$ M as compared with our equilibrium measurement of $K_d$ = 2 × $10^{-9}$ M using a nitrocellulose filter partitioning assay. TNfbg is one of two basic domains of an otherwise acidic protein that has an overall pI predicted to be <5. Notably, a previous U251 cell SELEX experiment[3] isolated a TN-C aptamer that binds the other basic region, which consists of fibronectin type III repeats 3–5. The two SELEX experiments differed in selection temperature (37 *versus* 4 °C) and oligonucleotide library (2'-F pyrimidine/2'-OH purine *versus* 2'-H), but it is not clear how these differences caused the current SELEX experiments to preferentially identify aptamers for the fibrinogen-like domain. It is known that distinct aptamers can bind different epitopes on a single-domain protein (56), and these data extend the observation by

[8] C. K. Lynott, data not shown.

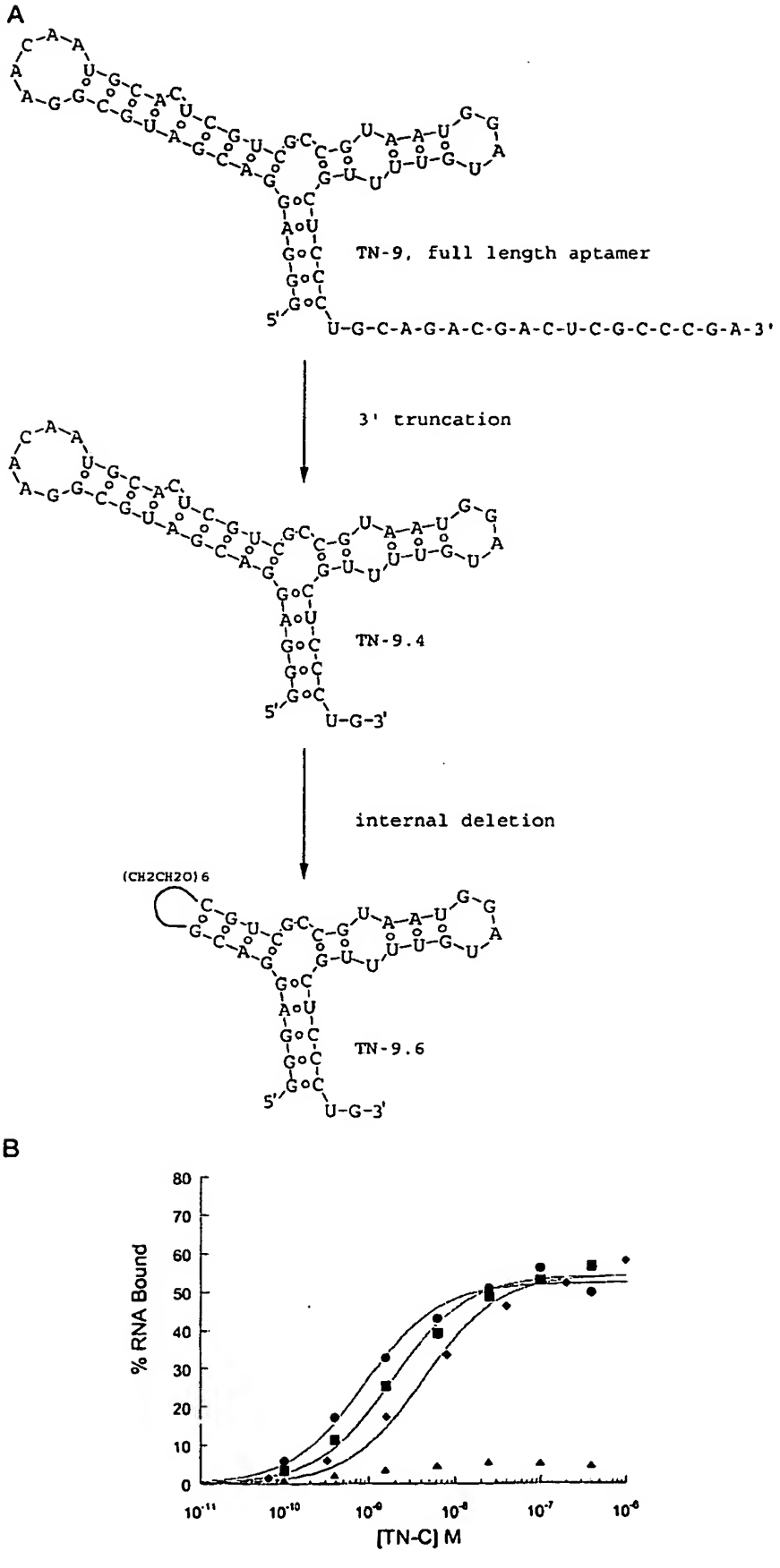


FIG. 8. **Aptamer size minimization.** A predicted secondary structure of TN-9 is the three-stem junction shown (*A*). Removal of 16 nucleotides from the 3′ end results in TN-9.4. An internal deletion of TN-9.4 replaced 17 nucleotides, $G^{10}$–$C^{27}$, with a single $(CH_2CH_2O)_6$ that was incorporated synthetically, producing TN-9.6. Aptamers were 5′ end-labeled with $^{32}P$, and affinity for tenascin-C was measured using a nitrocellulose filter partitioning assay. Data fits give $K_d = 1 \times 10^{-9}$ M (TN-9, *circles*), $2 \times 10^{-9}$ M (TN-9.4, *squares*), and $5 \times 10^{-9}$ M (TN-9.6, *diamonds*). No binding of tenascin-C to a random aptamer pool is evident (*triangles*).

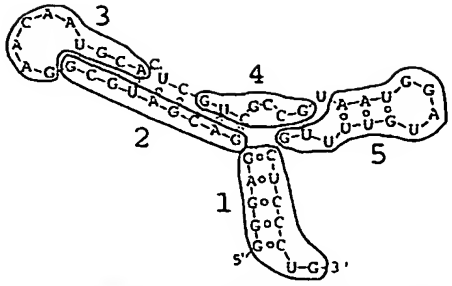


FIG. 9. **Sectoring of TN-9.4 for 2'-$OCH_3$ purine substitution.** For identification of purine positions that must remain 2'-OH for high affinity tenascin-C binding, aptamer TN-9.4 was arbitrarily divided into five sectors. Shown is a secondary structure predicted by an RNA folding algorithm. Sectors were chosen such that 2'-$OCH_3$ purine substitutions (sequences and affinities are shown in Table III) would occur on only one strand of a putative helix.

TABLE III

*TN-9.4: affinity of 2'-$OCH_3$-purine-substituted species*

To identify purines that cannot be substituted with 2'-$OCH_3$ purines, that aptamer was sectored into five areas as shown in Fig. 9. Aptamers were synthesized in which all purines in a sector were substituted with 2'-$OCH_3$ purines. Each aptamer was tested for tenascin-C affinity using a nitrocellulose filter partitioning assay. Having identified sectors showing affinity loss upon substitution, individual purines within the affected sectors were studied within the context of complete substitution in sectors 1, 3, and 5. The sum of these data was incorporated into a maximally 2'-$OCH_3$-substituted aptamer (in the context of the size-minimized 39-mer) to form TTA1, shown in Fig. 10.

| Substitution pattern | | | | | | $K_d$ for TN-C |
|---|---|---|---|---|---|---|
| Sector[a] | | | | | Nucleotide | |
| 1 | 2 | 3 | 4 | 5 | | |
| | | | | | | *nM* |
| | | | | | | 2 |
| x | | | | | | 6 |
| | x | | | | | 20 |
| | | x | | | | 7 |
| | | | x | | | >1000 |
| | | | | x | | 4 |
| x | x | x | x | x | | >1000 |
| x | | x | | x | | 2 |
| x | | x | | x | $G^6$ | 2 |
| x | | x | | x | $A^7$ | 2 |
| x | | x | | x | $G^9$ | 8 |
| x | | x | | x | $A^{10}$ | 1 |
| x | | x | | x | $G^{12}$,$G^{14}$ | 3 |
| x | | x | | x | $G^{28}$ | 37 |
| x | | x | | x | $G^{31}$ | 55 |
| x | | x | | x | $G^{34}$ | 7 |

[a] See Fig. 9.

indicating that conditions can also alter the dominant aptamer-binding domain on a large, multidomain protein.

Because reduced size may lead to increased tissue penetration rates (32) and will lead to more efficient chemical synthesis, we focused on size reduction of TN-9. Notably, an internal deletion was identified with the aid of an RNA structure prediction algorithm. This algorithm predicted that TN-9.4 could form a three-stem junction, among other possibilities, as a secondary structure. In comparing four family II aptamers, the algorithm revealed a potentially variant stem. We found that a deletion within this stem removed 17 nucleotides but left high-affinity binding intact. The resulting aptamer, TN-9.6, has a 5-fold reduced affinity relative to the full-length aptamer. Importantly, TN-9.6 is 17 nucleotides, or ~6 kDa, smaller than TN-9.4. The reduced size of TN-9.6 could increase tissue penetration rates and cause it to clear from the blood more rapidly than TN-9.4, which is an advantage for *in vivo* imaging.

To further stabilize the aptamer against nuclease activity, purines were substituted with 2'-$OCH_3$ groups. A combinatorial approach for identifying purine positions that tolerate 2'-$OCH_3$ substitution has been described (49). In that study (49), a library of partially 2-$OCH_3$-substituted aptamer molecules was synthesized. A selection experiment then separated binding from nonbinding species. The 2'-$OCH_3$ substitution pattern of the binding species was then identified by base hydrolysis, indicating purines that tolerate the substitution with retention of aptamer function. In our present work, the aptamer was sectored into five parts, and all purines in each sector were substituted, followed by affinity analysis. We found affinity reductions in two of five sectors and analyzed the contribution of individual nucleotides to affinity within these two sectors. In the end, 15 of the 19 purines could be substituted with 2'-$OCH_3$ without significant effect on affinity. Compared with the combinatorial 2'-$OCH_3$ substitution technique, a disadvantage of this sectoring approach is in the quantity of oligonucleotides that must be synthesized. The advantages are found in 1) obtaining direct binding data on a substituted species, rather than inferring effects on affinity from the selection experiment; and in 2) simpler oligonucleotide syntheses and binding experiments. Neither method requires knowledge of aptamer structure. After size reduction, 2'-$OCH_3$ substitution, 3' capping, and incorporation of a 5' amine, the synthetic aptamer TTA1 has a $K_d = 5 \times 10^{-9}$ M for binding to human TN-C, which is only a 5-fold reduction in affinity from the parent aptamer.

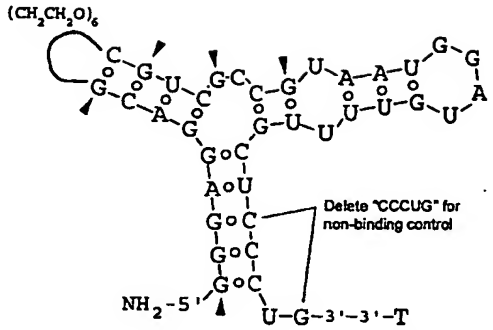


FIG. 10. **Aptamer TTA1: modifications and putative secondary structure.** All pyrimidines are 2'-F and all purines are 2'-$OCH_3$ except the Gs, marked by *arrowheads*, which are 2'-OH. $(CH_2CH_2O)_6$ is a phosphoramidite linker joining $G^9$ with $C^{27}$. Nucleotides 10–26 have been deleted. The oligonucleotide is synthesized with a 3'–3' linkage and a 5' primary amine incorporated by phosphoramidite coupling. TTA1.NB is a nonbinding sequence that is identical to TTA1 except that it contains a deletion in which CCCUG-3'-3'-T is replaced by 3'-3'-T. $K_d$ for tenascin-C = $5 \times 10^{-9}$ M.

Taken together, the expression pattern of TN-C, demonstrated binding to integrins, and demonstrated adhesive activities (1) suggest that TN-C may play an active role in tissue remodeling processes. Such processes include tumorigenesis, angiogenesis, atherosclerosis, and wound healing. In particular, recent data implicate the fibrinogen-like domain in binding to the integrin $\alpha_v\beta_3$ (25), a critical protein for angiogenesis. The aptamer described here binds tightly to the fibrinogen-like domain of TN-C and therefore has potential application in investigating the role of TN-C in tissue remodeling processes.

TTA1 is a size-minimized and nuclease-stabilized aptamer that binds with high affinity to tenascin-C, an abundant extracellular matrix protein that is overexpressed during tissue remodeling. The potential clinical advantages of aptamers for tissue targeting have been discussed (32). These advantages include high affinity and specificity, small size, amenity to chemical modification to alter biodistribution, and pharmacokinetics, and rapid tissue penetration. TTA1 can be conjugated to a variety of radioisotope chelators, fluorescent dyes, and

biologically active moieties. Thus modified, TTA1 can be tested for targeted delivery to the extracellular matrix of tumors and/or atherosclerotic lesions.

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# TENASCIN-C NUCLEIC ACID LIGANDS

## FIELD OF THE INVENTION

Described herein are high affinity nucleic acid ligands to tenascin-C. Also described herein are methods for identifying and preparing high affinity nucleic acid ligands to tenascin-C. The method used herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment. Further disclosed are high affinity nucleic acid ligands to tenascin-C. Further disclosed are RNA ligands to tenascin-C. Also included are oligonucleotides containing nucleotide derivatives chemically modified at the 2'-positions of the purines and pyrimidines. Additionally disclosed are RNA ligands to tenascin-C containing 2'-F and 2'OMe modifications. The oligonucleotides of the present invention are useful as diagnostic and/or therapeutic agents.

## BACKGROUND OF THE INVENTION

Tenascin-C is a 1.1-1.5 million Da, hexameric glycoprotein that is located primarily in the extracellular matrix. Tenascin-C is expressed during embryogenesis, wound healing, and neoplasia, suggesting a role for this protein in tissue remodeling (Erickson and Bourdon (1989) *Ann Rev Cell Biol* 5:71-92). Neoplastic processes also involve tissue remodeling, and tenascin-C is over-expressed in many tumor types including carcinomas of the lung, breast, prostate, and colon, astrocytomas, glioblastomas, melanomas, and sarcomas (Soini *et al.* (1993) Am J Clin Pathol 100(2):145-50; Koukoulis *et al.* (1991) Hum Pathol 22(7):636-43; Borsi *et al.* (1992) Int J Cancer 52(5):688-92; Koukoulis *et al.* (1993) J Submicrosc Cytol Pathol 25(2):285-95; Ibrahim *et al.* (1993) Hum Pathol 24(9):982-9; Riedl *et al.* (1998) Dis Colon Rectum 41(1):86-92; Tuominen and Kallioinen (1994) J Cutan Pathol 21(5):424-9; Natali *et al.* (1990) Int J Cancer 46(4):586-90; Zagzag *et al.* (1995) Cancer Res 55(4):907-14; Hasegawa *et al.* (1997) Acta Neuropathol (Berl) 93(5):431-7; Saxon *et al.* (1997) Pediatr Pathol Lab Med 17(2):259-66; Hasegawa *et al.* (1995) Hum Pathol 26(8):838-45). In addition, tenascin-C is overexpressed in hyperproliferative skin diseases, e.g. psoriasis (Schalkwijk *et al.* (1991) Br J Dermatol 124(1):13-20). and in atherosclerotic lesions (Fukumoto *et al.* (1998) *J* Atheroscler Thromb 5(1):29-35; Wallner *et al.* (1999) Circulation 99(10):1284-9). Radiolabeled antibodies that bind tenascin-C are used for imaging and therapy of tumors in clinical settings (Paganelli *et al.* (1999) Eur J Nucl Med 26(4):348-57; Paganelli *et al.* (1994) Eur J Nucl Med 21(4):314-21; Bigner *et al.* (1998) J Clin Oncol

16(6):2202-12; Merlo *et al.* (1997) Int J Cancer 71(5):810-6).

Aptamers against tenascin-C have potential utility for cancer diagnosis and therapy, as well as for diagnosis and therapy of atherosclerosis and therapy of psoriasis. Relative to antibodies, aptamers are small (7-20 kDa), clear very rapidly from blood, and are chemically synthesized. Rapid blood clearance is important for *in vivo* diagnostic imaging, where blood levels are a primary determinant of background that obscures an image. Rapid blood clearance may also be important in therapy, where blood levels may contribute to toxicity. SELEX technology allows rapid aptamer isolation, and chemical synthesis enables facile and site-specific conjugation of aptamers to a variety of inert and bioactive molecules. An aptamer to tenascin-C would therefore be useful for tumor therapy or *in vivo* or *ex vivo* diagnostic imaging and/or for delivering a variety of therapeutic agents complexed with the tenascin-C nucleic acid ligand for treatment of disease conditions in which tenascin-C is expressed.

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed the SELEX process, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. The SELEX process is a method for the in vitro evolution of nucleic acid molecules with highly specific binding to target molecules and is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned, United States Patent No. 5,475,096 entitled "Nucleic Acid Ligands," United States Patent No. 5,270,163 (see also WO 91/19813) entitled "Methods for Identifying Nucleic Acid Ligands," each of which is specifically incorporated by reference herein in its entirety. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands or aptamers, each having a unique sequence, and which have the property of binding specifically to a desired target compound or molecule. Each SELEX-identified nucleic acid ligand is a specific ligand of a given target compound or molecule. The SELEX process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can

serve as targets in the SELEX method. The SELEX method applied to the application of high affinity binding involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that nucleic acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by nucleic acids in biological systems.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and United States Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, United States Patent No. 5,763,177, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" and United States Patent Application Serial No. 09/093,293, filed June 8, 1998, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, which can be non-peptidic,

termed Counter-SELEX. United States Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified nucleic acid ligands containing modified nucleotides are described in United States Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent No. 5,580,737, supra, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-$NH_2$), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," now abandoned, describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chimeric SELEX," and United States Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes". Each of the above described patents and applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

## SUMMARY OF THE INVENTION

The present invention describes a method for isolating nucleic acid ligands that bind to tenascin-C with high specificity. Further described herein are nucleic acid ligands to tenascin-C. Also described herein are high affinity RNA ligands to tenascin-C. Further described are 2'fluoro-modified pyrimidine and 2'OMe-modified purine RNA ligands to tenascin-C. The method utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment. Included herein are the ligands that are shown in **Tables 3** and **4** and **Figure 10.**

Further included in this invention is a method for detecting the presence of a disease that is expressing tenascin-C in a biological tissue that may contain the disease. Still further included in this invention is a method for detecting the presence of a tumor that is expressing tenascin-C in a biological tissue that may contain the tumor. Further included in this invention is a complex for use in *in vivo* or *ex vivo* diagnostics. Still further included in this invention is a method for delivering therapeutic agents for the treatment or prophylaxis of diseased tissues that express tenascin-C. Still further included in this invention is a complex for use in delivering therapeutic agents for treatment or prophylaxis of diseased tissues that express tenascin-C.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows binding of Cell SELEX RNA pools to U251 cells.

Figure 2 shows proposed secondary structure of aptamers TTA1 and TTA1.NB. Included in the figure is the conjugation of the aptamers with Tc-99m chelator. All A's are 2'OMe modified. All G's, except as indicated, are 2'OMe modified. All C's and U's are 2'F modified.

Figure 3 shows images of U251 tumor xenografts in mice, obtained using Tc-99m-labeled TTA1 and TTA1.NB, three hours post-injection.

Figure 4 shows fluorescence microscopy of a U251 glioblastoma tumor section, taken three hours after i.v. injection of Rhodamine-Red-X-labeled TTA1.

Figure 5 shows the way in which the Tc-99m and linker is bound through the 5'G of TTA1.

Figure 6 describes TTA1/GS7641 uptake at 3 hours into various human tumor xenografts in mouse, compared to uptake of the non-binding control aptamer. ID/g = injected dose/gram.

Figure 7 displays the biodistribution of In-111 labeled TTA1/GS7641 using either DOTA or DTPA as the radiometal chelator, 3 hours after injection. ID/g = injected dose/gram.

Figure 8 shows the conjugation of the aptamer to DTPA. The $^{111}$In is shown as chelated by DTPA.

Figure 9 shows the conjugation of the aptamer to DOTA. The $^{111}$In is shown as chelated by DOTA.

Figure 10 shows the proposed secondary structure of aptamer TTA1. Included in the figure is the conjugation of the aptamer with Tc-99m chelator. The aptamer is shown in its Tc-99m labled form. All A's are 2'OMe modified. All G's, except as indicated, are 2'OMe modified. All C's and U's are 2'F modified.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The central method utilized herein for identifying nucleic acid ligands to tenascin-C is called the SELEX process, an acronym for Systematic Evolution of Ligands by Exponential enrichment and involves (a) contacting the candidate mixture of nucleic acids with tenascin-C (b) partitioning between members of said candidate mixture on the basis of affinity to tenascin-C, and c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to tenascin-C. The invention includes RNA ligands to tenascin-C. This invention further includes the specific RNA ligands to tenascin-C shown in **Tables 3** and **4** and **Figure 10**. More specifically, this invention includes nucleic acid sequences that are substantially homologous to and that have substantially the same ability to bind tenascin-C as the specific nucleic acid ligands shown in **Tables 3** and **4** and **Figure 10**. By substantially homologous it is meant a degree of primary sequence homology in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90%, 95%, or 99%. The percentage of homology as described herein is calculated as the percentage of nucleotides found in the smaller of the two sequences which align with identical nucleotide residues in the sequence being compared when 1 gap in a length of 10 nucleotides may be introduced to assist in that alignment. Substantially the same ability to bind tenascin-C means that the affinity is within one or two orders of magnitude of the affinity of the ligands described herein. It is well within the skill of those of ordinary skill in the art to determine whether a given sequence – substantially homologous to those specifically described herein – has the same ability to bind tenascin-C.

A review of the sequence homologies of the nucleic acid ligands of tenascin-C shown in **Tables 3** and **4** and **Figure 10** shows that sequences with little or no primary homology may have substantially the same ability to bind tenascin-C. For these reasons, this invention also includes nucleic acid ligands that have substantially the same postulated structure or structural motifs and ability to bind tenascin-C as the nucleic acid ligands shown in **Tables 3** and **4** and **Figure 10**. Substantially the same structure or structural motifs can be postulated by sequence alignment using the Zukerfold program (see Zuker (1989) Science 244:48-52). As would be known in the art, other computer programs can be used for predicting secondary structure and structural motifs. Substantially the same structure or structural motif of nucleic acid ligands in solution or as a bound structure can also be postulated using NMR or other techniques as would be known in the art.

Further included in this invention is a method for detecting the presence of a disease that is expressing tenascin-C in a biological tissue which may contain the disease by the method of (a) identifying a nucleic acid ligand from a candidate mixture of nucleic acids, the nucleic acid ligand being a ligand of tenascin-C, by the method comprising (i) contacting a candidate mixture of nucleic acids with tenascin-C, wherein nucleic acids having an increased affinity to tenascin-C relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; (ii) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture;
(iii) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids with relatively higher affinity and specificity for binding to tenascin-C, whereby a nucleic acid ligand of tenascin-C is identified; (b) attaching a marker that can be used in *in vivo* or *ex vivo* diagnostics to the nucleic acid ligand identified in step (iii) to form a marker-nucleic acid ligand complex; (c) exposing a tissue which may contain the disease to the marker-nucleic acid ligand complex; and (d) detecting the presence of the marker-nucleic acid ligand in the tissue, whereby a disease expressing tenascin-C is identified.

It is a further object of the present invention to provide a complex for use in *in vivo* or *ex vivo* diagnostics comprising one or more tenascin-C nucleic acid ligands and one or more markers. Still further included in this invention is a method for delivering therapeutic agents for the treatment or prophylaxis of disease conditions in which tenascin-C is expressed. Still further included in this invention is a complex for use in delivering therapeutic agents for treatment or prophylaxis of disease conditions in which tenascin-C is expressed.

Definitions

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided:

As used herein, "nucleic acid ligand" is a non-naturally occurring nucleic acid having a desirable action on a target. Nucleic acid ligands are often referred to as "aptamers." The target of the present invention is tenascin-C, hence the term tenascin-C nucleic acid ligand. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. Nucleic acid ligands are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a tenascin-C, by the method comprising: a) contacting the candidate mixture with tenascin-C, wherein nucleic acids having an increased affinity to tenascin-C relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture: and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids (see U.S. Patent Application No. 08/434,425, filed May 3, 1995, now United States Patent No. 5,789,157, which is hereby incorporated herein by reference).

As used herein, "candidate mixture" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

As used herein, "nucleic acid" means either DNA, RNA, single-stranded or double-stranded, and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge,

polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"SELEX" methodology involves the combination of selection of nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to tenascin-C.

The SELEX methodology is described in the SELEX Patent Applications.

"SELEX target" or "target" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this application, the SELEX target is tenascin-C.

"Complex" as used herein means the molecular entity formed by the covalent linking of one or more tenascin-C nucleic acid ligands with one or more markers. In certain embodiments of the present invention, the complex is depicted as A-B-Y, wherein A is a marker; B is optional, and comprises a linker; and Y is a tenascin-C nucleic acid ligand.

"Marker" as used herein is a molecular entity or entities that when complexed with the tenascin-C nucleic acid ligand, either directly or through a linker(s) or spacer(s), allows the detection of the complex in an *in vivo* or *ex vivo* setting through visual or chemical means. Examples of markers include, but are not limited to radionuclides, including Tc-99m, Re-188, Cu-64, Cu-67, F-18, $^{125}I$, $^{131}I$, $^{111}In$, $^{32}P$, $^{186}Re$; all fluorophores, including fluorescein, rhodamine, Texas Red; derivatives of the above fluorophores, including Rhodamine-Red-X; magnetic compounds; and biotin.

As used herein, "linker" is a molecular entity that connects two or more molecular entities through covalent bond or non-covalent interactions, and can allow spatial separation of the molecular entities in a manner that preserves the functional properties of one or more of the molecular entities. A linker can also be known as a spacer. Examples of a linker include, but are not limited to, the $(CH_2CH_2O)_6$ and hexylamine structures shown in **Figure 2.**

"Therapeutic" as used herein, includes treatment and/or prophylaxis. When used, therapeutic refers to humans and other animals.

"Covalent Bond" is the chemical bond formed by the sharing of electrons.

"Non-covalent interactions" are means by which molecular entities are held together by interactions other than Covalent Bonds including ionic interactions and hydrogen bonds.

In the preferred embodiment, the nucleic acid ligands of the present invention are derived from the SELEX methodology. The SELEX process is described in United States Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent No. 5,475,096, entitled "Nucleic Acid Ligands" and United States Patent No. 5,270,163, (see also WO 91/19813), entitled "Methods for Identifying Nucleic Acid Ligands." These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products which are nucleic acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired target compound or molecule. Target molecules are preferably proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX methodology can also be used to target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series of steps:

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, or

(c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and United States Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, United States Patent No. 5,763,177, entitled "Systematic

Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" and United States Patent Application Serial No. 09/093,293, filed June 8 1998, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," all describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent No. 5,496,938, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent No. 5,705,337 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent No. 5,637,459, supra, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-$NH_2$), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United

States Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," and United States Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

In United States Patent No. 5,496,938 methods are described for obtaining improved Nucleic Acid Ligands after the SELEX process has been performed. This patent, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," is specifically incorporated herein by reference.

United States Patent Application No. 08/434,425, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Tissue SELEX," filed May 3, 1995, now United States Patent No. 5,789,157, describes methods for identifying a nucleic acid ligands to a macromolecular component of a tissue, including cancer cells, and the nucleic acid ligands so identified. This patent is specifically incorporated herein by reference.

One potential problem encountered in the diagnostic or therapeutic use of nucleic acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the *in vivo* stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. See, e.g., U.S. Patent Application Serial No. 08/117,991, filed September 8, 1993, now abandoned, and United States Patent No. 5,660,985, both entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," which is specifically incorporated herein by reference. Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications

can also include 3' and 5' modifications such as capping. In preferred embodiments of the instant invention, the nucleic acid ligands are RNA molecules that are 2'-fluoro (2'-F) modified on the sugar moiety of pyrimidine residues.

The modifications can be pre- or post-SELEX process modifications. Pre-SELEX process modifications yield nucleic acid ligands with both specificity for their SELEX target and improved *in vivo* stability. Post-SELEX process modifications made to 2'-OH nucleic acid ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligand.

Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX process (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

The nucleic acid ligands of the invention are prepared through the SELEX methodology that is outlined above and thoroughly enabled in the SELEX applications incorporated herein by reference in their entirety.

The tenascin-C aptamers of the invention bind to the heparin binding site of the tenascin-C COOH terminus.

In certain embodiments of the present invention, the nucleic acid ligands to tenascin-C described herein are useful for diagnostic purposes and can be used to image pathological conditions (such as human tumor imaging). In addition to diagnosis, the tenascin-C nucleic acid ligands are useful in the prognosis and monitoring of disease conditions in which tenascin-C is expressed.

Diagnostic agents need only be able to allow the user to identify the presence of a given target at a particular locale or concentration. Simply the ability to form binding pairs with the target may be sufficient to trigger a positive signal for diagnostic purposes. Those skilled in the art would be able to adapt any tenascin-C nucleic acid ligand by procedures known in the art to incorporate a marker in order to track the presence of the nucleic acid ligand. Such a marker could be used in a number of diagnostic procedures, such as detection of primary and metastatic tumors and atherosclerotic lesions. The labeling markers exemplified herein are technetium-99m and $^{111}$In; however, other markers such as additional radionuclides, magnetic compounds, fluorophores, biotin, and the like can be conjugated to the tenascin-C nucleic acid ligand for imaging in an *in vivo* or *ex vivo* setting disease conditions in which tenascin-C is expressed (e.g., cancer, atherosclerosis, and psoriasis). The marker may be covalently bound to a variety of positions on the tenascin-C nucleic acid

ligand, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-position of a purine nucleotide, the hydroxyl group of the phosphate, or a hydroxyl group or other group at the 5' or 3' terminus of the tenascin-C nucleic acid ligand. In embodiments where the marker is technetium-99m of $^{111}$In, preferably it is bonded to the 5' or 3' hydroxyl of the phosphate group thereof or to the 5 position of a modified pyrimidine. In the most preferred embodiment, the marker is bonded to the 5' hydroxyl of the phosphate group of the nucleic acid ligand with or without a linker. In another embodiment, the marker is conjugated to the nucleic acid ligand by incorporating a pyrimidine containing a primary amine at the 5 position, and use of the amine for conjugation to the marker. Attachment of the marker can be done directly or with the utilization of a linker. In the embodiment where technetium-99m or $^{111}$In is used as the marker, the preferred linker is a hexylamine linker as shown in **Figure 10**.

In other embodiments, the tenascin-C nucleic acid ligands are useful for the delivery of therapeutic compounds (including, but not limited to, cytotoxic compounds, immune enhancing substances and therapeutic radionuclides) to tissues or organs expressing tenascin-C. Disease conditions in which tenascin-C may be expressed include, but are not limited to, cancer, atherosclerosis, and psoriasis. Those skilled in the art would be able to adapt any tenascin-C nucleic acid ligand by procedures known in the art to incorporate a therapeutic compound in a complex. The therapeutic compound may be covalently bound to a variety of positions on the tenascin-C nucleic acid ligand, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-position of a purine nucleotide, the hydroxyl group of the phosphate, or a hydroxyl group or other group at the 5' or 3' terminus of the tenascin-C nucleic acid ligand. In the preferred embodiment, the therapeutic agent is bonded to the 5' amine of the nucleic acid ligand. Attachment of the therapeutic agent can be done directly or with the utilization of a linker. In embodiments in which cancer is the targeted disease, 5-fluorodeoxyuracil or other nucleotide analogs known to be active against tumors can be incorporated internally into existing U's within the tenascin-C nucleic acid ligand or can be added internally or conjugated to either terminus either directly or through a linker. In addition, both pyrimidine analogues 2'2'-difluorocytidine and purine analogues (deoxycoformycin) can be incorporated. In addition, United States Application Serial No. 08/993,765, filed December 18, 1997, incorporated herein by reference in its entirety, describes, *inter alia*, nucleotide-based prodrugs comprising nucleic acid ligands directed to a

tumor, for example tenascin-C, for precisely localizing chemoradiosensitizers, and radiosensitizers and radionuclides and other radiotherapeutic agents to the tumor.

It is also contemplated that both the marker and therapeutic agent may be associated with the tenascin-C nucleic acid ligand, such that detection of the disease condition and delivery of the therapeutic agent is accomplished together in one aptamer or as a mixture of two or more different modified versions of the same aptamer. It is also contemplated that either or both the marker and/or the therapeutic agent may be associated with a non-immunogenic, high molecular weight compound or lipophilic compound, such as a liposome. Methods for conjugating nucleic acid ligands with lipophilic compounds or non-immunogenic compounds in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," which is incorporated herein in its entirety.

The therapeutic or diagnostic compositions described herein may be administered parenterally by injection (e.g., intravenous, subcutaneous, intradermal, intralesional), although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. They may also be applied locally by direct injection, can be released from devices, such as implanted stents or catheters, or delivered directly to the site by an infusion pump. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one embodiment, it is envisioned that the carrier and the tenascin-C nucleic acid ligand complexed with a therapeutic compound constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the tenascin-C nucleic acid ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic or diagnostic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing

tenascin-C nucleic acid ligands for systemic delivery may be via subcutaneous, intramuscular, intravenous, intraarterial, intranasal or vaginal or rectal suppository.

The following examples are provided to explain and illustrate the present invention and are not to be taken as limiting of the invention. Example 1 describes the materials and experimental procedures used in Example 2 for the generation of RNA ligands to tenascin-C. Example 2 describes the RNA ligands to tenascin-C and the predicted secondary structure of a selected nucleic acid ligand. Example 3 describes the determination of minimal size necessary for high affinity binding of a selected nucleic acid ligand, and substitution of 2'-OH purines with 2'-OMe purines. Example 4 describes the biodistribution of Tc-99m labeled tenascin-C nucleic acid ligands in tumor-bearing mice. Example 5 describes the use of a fluorescently labeled tenascin-C nucleic acid ligand to localize tenascin-c within tumor tissue. Example 6 describes detection of tumors *in vivo* by Aptamer TTA1 (also known as GS7641). Example 7 describes alternative labeling using $^{111}$In.

## EXAMPLES

**Example 1. Use of SELEX to obtain nucleic acid ligands to tenascin-C and to U251 glioblastoma cells.**

Materials and Methods

Tenascin-C was purchased from Chemicon (Temecula, CA). Single-stranded DNA-primers and templates were synthesized by Operon Technologies Inc. (Alameda, CA).

The SELEX-process has been described in detail in the SELEX Patent Applications. In brief, double-stranded transcription templates were prepared by Klenow fragment extension of 40N7a ssDNA:

5'- TCGCGCGAGTCGTCTG [40N] CCGCATCGTCCTCCC 3' (SEQ ID NO:1)

using the 5N7 primer:

5'-TAATACGACTCACTATAGGGAGGACGATGCGG-3' (SEQ ID NO:2)

which contains the T7 polymerase promoter (underlined). RNA was prepared with T7 RNA polymerase as described previously in Fitzwater and Polisky (1996) Methods Enzymol. 267: 275-301, incorporated herein by reference in its entirety. All transcription reactions were performed in the presence of pyrimidine nucleotides that were 2'-fluoro (2'-F) modified on the sugar moiety. This substitution confers enhanced resistance to ribonucleases that utilize the 2'-hydroxyl moiety for cleavage of the phosphodiester bond. Specifically, each transcription mixture contained 3.3 mM 2'-F UTP and 3.3 mM 2'-F CTP along with 1 mM

GTP and ATP. The initial randomized RNA library thus produced comprised $3 \times 10^{14}$ molecules. The affinities of individual ligands for tenascin-C were determined by standard methods using nitrocellulose filter partitioning (Tuerk and Gold (1990) Science 249(4968):505-10).

For each round of SELEX, Lumino plates (Labsystems, Needham Heights, MA) were coated for 2 hours at room temperature with 200 µl Dulbecco's PBS containing tenascin-C concentrations as shown in **Table 1**. After coating, wells were blocked using HBSMC+ buffer [20 mM Hepes, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 g/liter human serum albumin (Sigma, fraction V) for rounds 1 to 6 while for rounds 7 and 8 wells were blocked HBSMC+ buffer containing 1 g/liter casein (I-block; Tropix). Binding and wash buffer consisted of HBSMC+ buffer containing 0.05% Tween 20. For each SELEX round, RNA was diluted into 100 µl of binding buffer and allowed to incubate for 2 hours at 37°C in the protein coated wells that were pre-washed with binding buffer. After binding, six washes of 200 µl each were performed. Following the wash step, the dry well was placed on top of a 95°C heat block for 5 minutes. Standard AMV reverse transcriptase reactions (50 µl) were performed at 48°C directly in the well and the reaction products utilized for standard PCR and transcription reactions. Two synthetic primers 5N7 (see above) and 3N7a:

5'-TCGCGCGAGTCGTCTG-3' (SEQ ID NO:3)

were used for these template amplification and reverse transcription steps.

For cell SELEX, U251 human glioblastoma cells (Hum. Hered. (1971) 21:238) were grown to confluence in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD) on six-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) and washed three times using Dulbecco's PBS supplemented with $CaCl_2$ (DPBS, GIBCO BRL) buffer. RNA labeled internally by transcription (Fitzwater (1996) *supra*) was incubated with the cells at 37°C for one hour. The labeled RNA was then removed, and the cells were washed six times for ten minutes each at 37°C with DPBS. DPBS containing 5 mM EDTA was then added and incubated with the cells for 30 minutes to elute bound RNAs that remained after the washing steps. This RNA was quantitated by a standard liquid scintillation counting protocol and amplified using RT-PCR.

Binding assays for the U251 cells. Internally labeled RNA was incubated at increasing concentrations with confluent U251 cells in six-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C for 60 min. Unbound RNA was washed away

using three 10 minute washes with DPBS+ $CaCl_2$ at 37°C, and bound RNA was collected by disrupting the cells using Trizol (Gibco BRL, Gaithersburg, MD). Bound RNA was quantitated by liquid scintillation counting.

Cloning and Sequencing. Amplified affinity enriched oligonucleotide pools were purified on an 8% polyacrylamide gel, reverse transcribed into ssDNA and the DNA amplified by the polymerase chain reaction (PCR) using primers containing BamH1 and HindIII restriction endonuclease sites. PCR fragments were cloned, plasmids prepared and sequence analyses performed according to standard techniques (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed. 3 vols., Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

**Example 2. RNA ligands to tenascin-C.**

Nucleic Acid Ligands to U251 cells were obtained by the SELEX process and are described in United States Patent Application Serial No. 08/434,425, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Tissue SELEX," filed May 3, 1995, now United States Patent No. 5,789,157. Subsequently it was determined that the ligands that were obtained were tenascin-C nucleic acid ligands.

To obtain oligonucleotide ligands against human tenascin-C, eight rounds of SELEX were performed using the randomized nucleotide library as described above in Materials and Methods. RNA and protein input into each round is shown in **Table 1**. After 8 rounds of SELEX, the affinity of the oligonucleotide pool for tenascin-C was 10 nM, and this affinity did not increase with additional SELEX rounds.

To obtain ligands to U251 glioblastoma cells, nine rounds of SELEX were performed using the randomized nucleotide library. After nine rounds of binding to U251 cells and EDTA elution, rounds 3, 5 and 9 were tested for their ability to bind to U251 cells. **Figure 1** shows that as the number of SELEX rounds increases, the amount of bound RNA also increases at a particular concentration. Because of the complexity of the target tissue, it was not possible to estimate the affinity of the oligonucleotide pools for the unknown target molecules(s) on these cells.

The E9 pool (nine rounds of binding and EDTA elution from U251 cells) was then used as a starting point for a SELEX against purified tenascin-C. Two rounds of SELEX using purified tenascin-C were performed as described above. Input protein and RNA concentrations for two rounds of SELEX (E9P1 and E9P2) are described in **Table 2**.

In summary, three different SELEX experiments were performed: an experiment using purified tenascin-C as the target, an experiment using U251 glioblastoma cells as the target, and an experiment in which the SELEX pool from the U251 glioblastoma cells was used to initiate a SELEX experiment using purified tenascin-C as the target.

All three SELEX experiments were analyzed by cloning and sequencing ligands from round 8 of the purified tenascin-C SELEX ("TN" sequences), from round 9 of the U251 cell SELEX ("E9" sequences), and from round 2 of the U251/tenascin-C hybrid SELEX ("E9P2" sequences). The sequences of 34 unique clones are shown in **Table 3**, and are divided into two major groups: tenascin-C ligands ("TN" and "E9P2" sequences) and U251 cell ligands ("E9" ligands). Among the tenascin-C ligands, the majority of the clones (65 total) represent one of two distinct sequence classes designated Family I and Family II (**Figure 1**). Examination of the variable region of the 12 clones in Family I revealed 7 unique sequences that are related through the consensus sequence GACNYUUCCNGCYAC (SEQ ID NO:12). Examination of the variable region of the 18 clones in Family II revealed sequences that share a consensus sequence CGUCGCC (**Table 3**). The E9 sequences could be grouped into a related set by virtue of conserved GAY and CAU sequences within the variable regions. The remaining sequences did not appear related to other sequences and were classified as orphans. Three sequences predominate, with E9P2-1, E9P2-2, and TN9 represented 14,16, and 10 times respectively. In the "Orphan" category, one sequence, TN18, was represented twice. Overall, these data represent a highly enriched sequence pool.

Most individuals displayed low nanomolar dissociation constants, with the three most prevalent sequences, TN9 and E9P2-1 and –2, having the highest affinities at 5 nM, 2nM, and 8 nM. (**Table 3**). These results indicate that the U251 cell SELEX is a repository for aptamers against tenascin-C, and that only two rounds of SELEX were required to isolate the tenascin-specific ligands from the cell SELEX pool. Oligonucleotide ligands against other proteins can be similarly isolated from the E9 pool using purified protein targets.

**Example 3. Determination of minimal size of TN9, and substitution of 2'-OH purines with 2'-OMe purines: synthesis of aptamer TTA1.**

Oligonucleotide synthesis procedures were standard for those skilled in the art (Green *et al.* (1995) Chem Biol 2(10):683-95). 2'-fluoro pyrimidine phosphoramidite monomers were obtained from JBL Scientific (San Luis Obispo, CA); 2'-OMe purine, 2'-OH purine, hexylamine, and $(CH_2CH_2O)_6$ monomers, along with the dT polystyrene solid support, were

obtained from Glen Research (Sterling, VA). Aptamer affinities were determined using nitrocellulose filter partitioning (Green *et al.*, supra).

TN9 was chosen for further analysis based on its high affinity for tenascin-C. We first searched for a minimal sequence necessary for high affinity binding. Using standard techniques (Green et al, *supra*), it was discovered that nucleotides 3'of nucleotide 55 were required for binding to tenascin-C, while no nucleotides could be removed from the 5' end without loss of affinity. To further decrease the TN9's length from 55 nucleotides and retain high affinity binding, we then attempted to define internal deletions of TN9. The first 55 nucleotides of TN9, along with the first 55 nucleotides of related family II ligands TN7, TN21, and TN41, were input into a computer algorithm to determine possible RNA secondary structure foldings (mfold 3.0, accessed at http://www.ibc.wustl.edu/~zuker/. M. Zuker, D.H. Mathews & D.H. Turner. Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In: RNA Biochemistry and Biotechnology, J. Barciszewski & B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, (1999)). Among many potential RNA foldings predicted by the algorithm, a structure common to each oligonucleotide was found. This structure, represented by oligonucleotide TTA1 in **Figure 2**, contains three stems that meet at a single junction, a so-called 3-stem junction. This folding places the most highly conserved nucleotides of family II oligonucleotides at the junction area. In comparing TN9, TN7, TN21, and TN41, the second stem was of variable length and sequence, suggesting that extension of the second stem is not required for binding to tenascin-C. Testing this hypothesis on TN9, we found that nucleotides 10-26 could be replaced with an ethylene glycol linker, $(CH_2CH_2O)_6$. The linker serves as a substitute loop and decreases the size of the aptamer. Additionally, four-nucleotide loops (CACU or GAGA) that replace nucleotides 10-26 produce sequences with high affinity for tenascin-C. It would be well within one skilled in the art to determine other nucleotide loops or other spacers that could replace nucleotides 10-26 to produce sequences with high affinity for tenascin-C.

To increase protection against nuclease activity, purine positions that could be substituted with the corresponding 2'-OMe purines were located. The oligonucleotide was arbitrarily divided into five sectors and all purines within each sector were substituted by the corresponding 2'-OMe purine nucleotide, a total of five oligonucleotides (**Table 4**, Phase I syntheses). The affinity of each oligonucleotide for tenascin-C was determined, and it was found that all purines within sectors 1,3 and 5 could be substituted without appreciable loss in

affinity. Within sectors 2 and 4, individual purines were then substituted with 2'-OMe purines and the effect of affinity was measured (**Table 4, Phase III syntheses**). From these experiments, it was deduced that substitution of nucleotides G9, G28, G31, and G34, with 2'-OMe G causes loss in affinity for tenascin-C. Therefore these nucleotides remain as 2'-OH purines in the aptamer TTA1.

The aptamer TTA1 (**Table 4**) was then synthesized with the $(CH_2CH_2O)_6$ (Spacer 18) linker, a 3'-3' dT cap for exonuclease protection, a 5' hexylamine (**Table 4**), and all purines as 2'-OMe except the 5 Gs indicated in **Table 4**. A non-binding control aptamer, TTA1.NB, was generated by deleting 5 nucleotides at the 3' end to produce TTA1.NB. TTA1 binds to tenascin-C with an equilibrium dissociation constant ($K_d$) of 5 nM, while TTA1.NB has a $K_d$ of > 5 μM for tenascin-C.

Nucleotides 10-26 can be replaced by a non-nucleotide ethylene glycol linker. It is therefore likely that TTA1 can be synthesized in two separate pieces, where a break is introduced at the position of the ethylene glycol linker and new 5' and 3' ends are introduced. Subsequent to synthesis, the two molecules will incubated together to allow hybrid formation. This method allows introduction of additional amine groups as well as nucleotides at the new 5' and 3' ends. The new functionalities could be used for bioconjugation. In addition, two-piece synthesis results in increased chemical synthetic yield due to shortening the length of the molecules.

**Example 4. Biodistribution of Tc-99m labeled aptamers in tumor-bearing mice.**

Aptamer biodistribution was tested by conjugating a Tc-99m chelator ($Hi_{15}$: Hilger *et al.* (1998) Tet Lett 39:9403-9406) to the 5' end of the oligonucleotide as shown in **Figure 2**, and radiolabeling the aptamer with Tc-99m. The aptamer in its Tc-99m labeled form is shown in **Figure 10**. TTA1 and TTA1.NB were conjugated to $Hi_{15}$ at 50 mg/ml aptamer in 30% dimethylformamide with 5 molar equivalents of $Hi_{15}$-N-hydoxysuccinimide, buffered in 100 mM Na Borate pH 9.3, for 30 minutes at room temperature. The aptamer in its Tc-99m labeled form is shown in **Figure 10**. Reversed phase HPLC purification yielded $Hi_{15}$-TTA1 and $Hi_{15}$-TTA1.NB. The oligonucleotides were then labeled with Tc-99m in the following manner: to 1 nmole Hi15-aptamer was added 200 μL of 100 mM sodium phosphate buffer, pH 8.5, 23 mg/mL NaTartrate, and 50 μL Tc-99m pertechnetate (5.0 mCi) eluted from a Mo-99 column (Syncor, Denver) within 12 hours of use. The labeling reaction was initiated by

the addition of 10 μL 5 mg/mL $SnCl_2$. The reaction mixture was incubated for 15 minutes at 90° C. The reaction was separated from unreacted Tc-99m by spin dialysis through a 30,000 MW cut-off membrane (Centrex, Schleicher & Scheull) with two 300 μL washes. This labeling protocol results in 30-50% of the added 99mTc being incorporated with a specific activity of 2-3 mCi/nmole RNA. The Tc-99m is bound through the 5'G as shown in **Figure 5.**

For biodistribution experiments, U251 xenograft tumors were prepared as follows: U251 cells were cultured in Dulbeccos' Modified Eagle's Medium supplemented with 10% v/v fetal calf serum (Gibco BRL, Gaithersburg, MD). Athymic mice (Harlan Sprague Dawley, Indianapolis, IN) were injected subcutaneously with $1x10^6$ U251 cells. When the tumors reached a size of 200-300 mg (1-2 weeks), Tc-99m labeled aptamer was injected intravenously at 3.25 mg/kg. At indicated times, animals were anesthetized using isoflurane (Fort Dodge Animal Health, Fort Dodge, IA), blood was collected by cardiac puncture, and the animal was sacrificed and tissues were harvested. Tc-99m levels were counted using a gamma counter (Wallac Oy, Turku, Finland). Aptamer uptake into tissues was measured as the % of injected dose per gram of tissue (%ID/g).

Images of mice were obtained using a gamma camera. Mice were placed onto the camera (Siemens, LEM+) under anesthesia (isoflurane). Data were collected (30 sec to 10 minutes) and analyzed using Nuclear MAC software version 3.22.2 (Scientific Imaging, CA) on a Power MAC G3 (Apple Computer, CA).

Biodistribution experiments, **Table 5**, indicated rapid and specific uptake of the aptamer into tumor tissue; the non-binding aptamer does not remain in the tumor. Blood levels of Tc-99m also cleared rapidly. After three hours, Tc-99m levels brought into the tumor using $Hi_{15}$-TTA1 had a very long half life (> 18 hrs). This indicates that once the aptamer penetrates the tumor, the radiolabel carried with it remains in the tumor for long periods of time. Such data indicate that cytotoxic agents, including radionuclides and non-radioactive agents, conjugated to the aptamer will also remain in the tumor with long half lives.

Tc-99m radioactivity also appears in other tissues, notably the small and large intestines. The hepatobiliary clearance pattern seen here can be readily altered by those skilled in the art, for example by altering the hydrophilicity of the Tc-99m chelator, changing the chelator, or changing the radiometal/chelator pair altogether.

Whole animal images were obtained using Tc-99m labeled $Hi_{15}$-TTA1 and at 3 hours post-injection. Images obtained from mice injected with $Hi_{15}$-TTA1, but not from mice injected with $Hi_{15}$-TTA1.NB, clearly show the tumor (**Figure 3**). Additional radioactivity is evident in gastrointestinal tract, as predicted by the biodistribution experiments.

**Example 5. Use of fluorescently labeled TTA1 to localize tenascin-C within tumor tissue.**

Materials and Methods.

TTA1 and TTA1.NB were synthesized as described above. Succinimdyl Rhodamine-Red-X (Molecular Probes, Eugene, OR) was conjugated to the 5' amine of the aptamers as described above for $H_{15}$-NHS conjugation. The Rhodamine-Red-X-conjugated aptamers, TTA1-Red and TTA1.NB-Red, were purified by reversed phase HPLC. U251 cell culture and tumor growth in nude mice were as described above. Five nmol of TTA1-Red or TTA1.NB-Red were injected intravenously into nude mice and at the desired time the animal was placed under anesthesia, perfused with 0.9% NaCl, and sacrificed. The tumor was excised and placed in formalin. After 24 hr in formalin, 10 μM sections were cut and Rhodamine-Red-X was detected using a fluorescence microscope (Eclipse E800, Nikon, Japan).

Results: TTA1-Red has identical affinity for tenascin-C as the unconjugated parent aptamer, TTA1, at 5 nM. We compared tumor fluorescence levels of TTA1-Red and TTA1.NB-Red 10 min post-injection. The binding aptamer, TTA1-Red, strongly stains the tumor but not adjacent tissue (**Figure 4**). In contrast, only tissue auto-fluorescence is detected with TTA1.NB-Red. These results demonstrate the utility of the aptamer in fluorescent detection of tenascin-C *in vivo*, and the aptamer may be similarly used for staining tissues sections *ex vivo*.

**Example 6. Detection of Tumors *In Vivo* by Aptamer TTA1 (now also known as GS7641): additional tumor types.**

Aptamer labeling, biodistribution, and nude mouse tumor xenografts were performed as described in Example 4.

Many human tumor types are known to express tenascin-C. To assess the ability of TTA1/GS7641 to target tumor types in addition to glioblastomas, human tumor cell lines were grown as tumors in nude mice. Tumor tissue was tested for expression of human

tenascin-C, and those tumors expressing human tenascin-C were tested for aptamer uptake. **Figure 6** demonstrates aptamer uptake in several tumors, including glioblastoma, breast, colorectal, and rhabdomyosarcoma. Specific uptake into the tumor is demonstrated by the comparison between binding (TTA1/GS7641) and non-binding aptamer (TTA1.NB). Note that KB, a xenograft that expresses mouse but not human TN-C, does not show tumor uptake. This experiment extends the observation of glioblastoma uptake into additional carcinomas and sarcomas, and further indicates that all tumors expressing human tenascin-C show uptake of TTA1/GS7641.

**Example 7. Alternative Labeling Using In-111.**

Tumor xenograft and biodistribution studies were performed as described in Example 4. To couple DTPA and DOTA to TTA1/GS7641, the cyclic anhydride of each was incubated with the amine-containing TTA1/GS7641 under neutral pH conditions using standard methods. The structures of the DTPA and conjugates are shown in **Figure 8** and **Figure 9**, respectively, where each has been labeled with In-111. The DOTA conjugate has the identical linkers as for DTPA. DOTA- and DTPA-conjugates were labeled with In-111 by incubation at 95°C in 0.5 M NaOAc, pH 5.5 for 30 min. After removal of unincorporated radiolabel by spin dialysis over a 30K cut-off membrane, radiolabeled aptamer was transferred into phosphate-buffered saline for injection into tumor-bearing mice.

The biodistribution of In-11 labeled aptamer is markedly different from the Tc-labeled formulation described in Example 4. **Figure 7** shows that, relative to Tc-99m-labeled TTA1/GS7641, In-111-labeled TTA1/GS7641 radioactivity in the intestines is greatly reduced, with a concomitant increase in liver and kidney uptake. This experiment indicates that the chemical properties of the chelator have a large effect on distribution of the radiolabel of TTA1/GS7641 within a living animal. Biodstribution patterns that are different from that of Hi15-TTA1/GS7641 may useful for targeting tumors under certain clinical conditions where hepatobiliary clearance is undesired. Such conditions include but are not limited to radiotherapy applications as well as imaging of intestines, prostate and other abdominal regions.

Table 1. Tenascin-C SELEX RNA and protein input.

| | Tenascin-C | RNA |
|---|---|---|
| Round | (pMol/well) | (pMol/well) |
| 1 | 12 | 200 |
| 2 | 12 | 200 |
| 3 | 12 | 200 |
| 4 | 12 | 200 |
| 5 | 2 | 33 |
| 6 | 2 | 33 |
| 7 | 2 | 33 |
| 8 | 0.2 | 3.3 |

Table 2. Cell SELEX/tenascin-C SELEX RNA and protein input

| | Tenascin-C | RNA |
|---|---|---|
| Round | (pMol/well) | (pMol/well) |
| E9P1 | 2 | 33 |
| E9P2 | 2 | 33 |

Table 3: Tenascin-C Sequences: purified protein SELEX (tenascin sequences) and U251 cell SELEX+purified protein SELEX (E9P2 sequences)

**Family I**

| | SEQ ID NO: | | | |
|---|---|---|---|---|
| TN11 | 4 | ggGAggAcGauGcgg | CAAUcAAAACUcACGUUA UUCCC UCAUCUAUUAGCUUCCC cagacgacucgcccga | 10 nM |
| TN45 | 5 | gggaggacgaugcgg | CAAUCUcCGAAAAAGACUCUUCCU GCAUCCUCUcACCCCC cagacgacucgcccga | 30 nM |
| TN4 | 6 | gggaggacgaugcgg | CAACCUc GAAAGACUUUUCCC GCAUCACUGUGUACUCCCC cagacgacucgcccga | 40 nM |
| TN22 | 7 | gggaggacgaugcgg | CAACCUc GAUAGACUUUUCCC GCAUCACUGUGUACUCCCC cagacgacucgcccga | 40 nM |
| TN32(2) | 8 | gggAggAcGauGcgg | cAaCCUcAA UCUuGaCAUUUCCC GcACCUAAAUUUG CCCC cagacgacucgcccga | 15 nM |
| TN14 | 9 | gggaggacgaugcgg | CAAACGAUC ACU UACCUUUCCU GCAUCUGCUAGC CUCCCC cagacgacucgcccga | 20 nM |
| TN44(3) | 10 | gggaggacgaugcgg | ACGCCAGCCAUUGACCCUCGCUUCCACUAUUCCAUCCCCC cagacgacucgcccga | 10 nM |
| TN29(2) | 11 | gggaggacgaugcgg | CCAACCUCAUUUUGACACUUCGCCGCACCUAAUUGCCCC cagacgacucgcccga | 25 nM |
| consensus: | 12 | | GACNYUUCCN GCAYC | |

**Family II**

| | | | | |
|---|---|---|---|---|
| E9P2-4(5) | 13 | gggaggacgaugcgg | AACCCAUA ACGCGA ACCGACCAACAUGCCUCCCGUGCCCC cagacgacucgcccga | |
| E9P2-1(14) | 14 | gggAggacgaugcgg | UGCCCAUAG AAGCGU GCCGCUAAUGCUAACGCCCUCCCC cagacgacucgcccga | 2 nM |
| E9P2-2(16) | 15 | gggaggacgaugcgg | UGCCCACU AUGCGU GCCGAAAAACAUUUCCCCCUCUACCC cagacgacucgcccga | 8 nM |
| TN7 (3) | 16 | gggaggacgaugcgg | AACACUUUCCCAUGCGUCGCC AUACC GGAUAUAUUGCUCC cagacgacucgcccga | 20 nM |
| TN21(4) | 17 | gggaggacgaugcgg | ACUGGACCAAACCGUCGCCGAUACCCGGAUACUUUGCUCC cagacgacucgcccga | 10 nM |
| TN9(10) | 18 | gggaggacgaugcgg | AACAAUGCACUCGUCGCCGUAAU GGAUGUUUUGCUCCCUG cagacgacucgcccga | 5 nM |
| TN41 | 19 | gggaggacgaugcgg | UUAAGUCUCGGUUGAAU GCCCAUCCC AGAUCCCCCUGACC cagacgacucgcccga | 20 nM |
| consensus: | | | GCGUCGCCG | |

**Orphans**

| | | | | |
|---|---|---|---|---|
| E9P2-17 | 20 | gggaggacgaugcgg | AUGGCAAGUCGAACCAUCCCCCACGCUUCUCCUGUUCCCC cagacgacucgcccga | |
| E9P2-48 | 21 | gggaggacgaugcgg | GAAGUUUUcUCUGCCUUGGUUUCGAUUGGCGCCUccCCCC cagacgacucgcccga1 | |
| E9P2-14 | 22 | gggaggacgaugcgg | UCGAGCGgUCGACCGUCAACAAGAAUAAAGCGUGUCCCUG cagacgacucgcccga | |
| E9P2-17 | 23 | gggaggacgaugcgg | AUGGCAAGUCGAACCAUCCCCCACGCUUCUCCUGUUCCCC cagacgacucgcccga | |
| E9P2-22 | 24 | gggaggacgaugcgg | ACUAGACcgCGAGUCCAUUCAACUUGCCCAAAAaAAAACcUCCCC cagacgacucgcccga | |
| E9P2-40 | 25 | gggaggacgaugcgg | GAGAUCAACAUUCCUCUAGUUUGGUUCCAACCUACACCCC cagacgacucgcccga | |
| E9P2-41 | 26 | gggaggacgaugcgg | ACGAGCGUCUCAUGAUCACACUAUUUCGUCUCAGUGUGCA cagacgacucgcccga | |
| TN18 | 27 | gggaggacgaugcgg | UCGACCUCGAAUGACUCUCCACCUAUCUAACAUCCCCCCC cagacgacucgcccga | 145 nM |

Table 3 (Continued)

| | | | | |
|---|---|---|---|---|
| TN20 | 28 | gggaggacgaugcgg | UCGACCUCGAAUGACUCUCCACCUAUCUAACAGCCUUCCC cagacgacucgcccga | |
| TN51 | 29 | gggaggacgaugcgg | AGAACUCAUCCUAACCGCUCUAACAAAUCUUGUCCGACCG cagacgacucgcccga | |
| TN8 | 30 | gggaggacgaugcgg | AUAAUUcGACACCAACCAGGUCCCGGAAAUCAUCCCUCUG cagacgacucgcccga | >10 uM |
| TN27 | 31 | gggaggacgaugcgg | AAACCAACCGUUGACCAC CUUUUCGUUUCCGGAAAGUCCC cagacgacucgcccga | 110 nM |
| TN39 | 32 | gggaggacgaugcgg | AAGCCAACCCUCUAGUCAGCCUUUCGUUUCCCACGCCACC cagacgacucgcccga | |
| TN24 | 33 | gggaggacgaugcgg | gACCAACUAAACUGUUCGAAAGCUGGaACAUGUCCUGACGC cagacgacucgcccga | 10 nM |
| TN5 | 34 | gggaggacgaugcgg | ACCAACUAAACUGUUCGAAAGCUGGAACACGUCCUGACGC cagacgacucgcccga | |
| TN36 | 35 | gggaggacgaugcgg | ACCAACUAAACUGUUCGAAAGCUAGAACACGUCCAGACGC cagacgacucgcccga | |
| TN6 | 36 | gggaggacgaugcgg | ACCAACUAAACUGUUCGAAAGCUGGAACACGUUCUGACGC cagacgacucgcccga | |
| TN10 | 37 | gggaggacgaugcgg | ACCAACUAAACUGUUCGAAAGCUGGAAUACGUCCUGACGC cagacgacucgcccga | |
| TN1 | 38 | gggaggacgaugcgg | AAGUUUA GuGCUCCAGUUCCGACACUCCUcUACUCAGCCC cagacgacucgcccga | >10 uM |
| TN109 | 39 | gggaggacgaugcgG | AgCCAGAGCCUcUcUcAGUUcUaCAGAACUuACCcACUGG cagacgacucgcccga | |
| TN110 | 40 | gggaggacgaugcgg | ACCUAACUCAAUCAGGAACCAAACCUAGCACUCUCAUGGC cagacgacucgcccga | |

U251 SELEX Aptamers, EDTA Elution (E9)

| | | | |
|---|---|---|---|
| E9-8 (3) | 41 | gggaggacgaugcgg | GAGAUCAACAUUCCUCUAGUUUGGUUCCAACCUACACCCC cagacgacucgcccga |
| E9-15 | 42 | gggaggacgaugcgg | AUCUCGAUCCUUCAGCACUUCAUUUCAUUCCUUUcUGCCC cagacgacucgcccga |
| E9-6 | 43 | gggaggacgaugcgg | ACGAUCCUUUCCUUAA CAUUUCAUCAUUUCUCUUGUGCCC cagacgacucgcccga |
| E9-5(2) | 44 | gggaggacgaugcgg | UGACGACAACUCGACUG CAUAUCUCACAACUCCUGUGCCC cagacgacucgcccga |
| E9-3 (6) | 45 | gggaggacgaugcgg | ACUAGACCGCGAGUC CAUUCAACUUGCCCAAAAACCUCCCC cagacgacucgcccga |
| E9-9 | 46 | gggaggacgaugcgg | GCGCAUCGAGCAACAUCCGAUUCGGAUUCCUCCACUCCCC cagacgacu gcccga |

Table 4: 2'-OMe Substitutions, Internal Deletions, TTA1, and TTA1.NB

**Phase I. 2'-OMe. Affinity.**

| Sequence | SEQ ID NO: | | Kd . |
|---|---|---|---|
| TN9.3 | 47 | gggaggacgaugcggAACAAUGCACUCGUCGCCGUAAUGGAUGUUUUGCU5 | >10 uM |
| TN9.4 | 48 | GGGAGGACGAUGCGGAACAAUGCACUCGUCGCCGUAAUGGAUGUUUUGCUCCCUG5 | 2 nM |
| TN9.4M1 | 49 | 66676GACGAUGCGGAACAAUGCACUCGUCGCCGUAAUGGAUGUUUUGCUCCCU65 | 6 nM |
| TN9.4M2 | 50 | GGGAG67C67U6C6GAACAAUGCACUCGUCGCCGUAAUGGAUGUUUUGCUCCCUG5 | 20 nM |
| TN9.4M3 | 51 | GGGAGGACGAUGCG677C77U6C7CUCGUCGCCGUAAUGGAUGUUUUGCUCCCUG5 | 7 nM |
| TN9.4M4 | 52 | GGGAGGACGAUGCGGAACAAUGCACUC6UC6CC6UAAUGGAUGUUUUGCUCCCUG5 | nb |
| TN9.4M5 | 53 | GGGAGGACGAUGCGGAACAAUGCACUCGUCGCCGU77U667U6UUUU6CUCCCUG5 | 4 nM |
| TN9.4Me | 54 | 6667667C67U6C6677C77U6C7CUC6UC6CC6U77U667U6UUUU6CUCCCU65 | 10 nM |

6=mG; 7=mA; 5=3'-3' Cap, 1 = hexylamine

**Phase III. 2'-OMe. Affinity.**

| | | | |
|---|---|---|---|
| TN9.4M1235 | 55 | 16667667C67U6C6677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 16.5 nM |
| TN9.4M135G6 | 56 | 166676ACGAUGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 2.2 nM |
| TN9.4M135A7 | 57 | 166676G7CGAUGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 1.7 nM |
| TN9.4M135G9 | 58 | 166676GAC6AUGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 7.7 nM |
| TN9.4M135A10 | 59 | 166676GACG7UGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 1.3 nM |
| TN9.4M135G12G14 | 60 | 166676GACGAU6C6677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 2.5 nM |
| TN9.4M135G28 | 61 | 166676GACGAUGCG677C77U6C7CUC6UCGCCGU77U667U6UUUU6CUCCCU65 | 37 nM |
| TN9.4M135G31 | 62 | 166676GACGAUGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 55 nM |
| TN9.4M135G34 | 63 | 166676GACGAUGCG677C77U6C7CUCGUCGCCGU77U667U6UUUU6CUCCCU65 | 7 nM |
| TTA1: | 64 | 5'-1G667667CG-$(CH_2CH_2O)_6$-CGUCGCCGU77U667U6UUUU6CUCCCU65 | 5 nM |
| TTA1.NB: | 65 | 5'-1G667667CG-$(CH_2CH_2O)_6$-CGUCGCCGU77U667U6UUUU6CU5 | >5 uM |

Table 5: Biodistribution of Tc-99m-TTA1 and -TTA1.NB

| | min | TTA1 | TTA1.NB | | min | TTA1 | TTA1.NB |
|---|---|---|---|---|---|---|---|
| tumor | 2 | 4.470 ± 0.410 | 4.510 ± 0.300 | kidney | 2 | 44.430 ± 4.280 | 54.470 ± 1.210 |
| | 10 | 5.940 ± 0.590 | 3.020 ± 0.210 | | 10 | 18.810 ± 0.940 | 14.320 ± 2.080 |
| | 60 | 2.689 ± 0.310 | 0.147 ± 0.018 | | 60 | 1.514 ± 0.040 | 0.637 ± 0.111 |
| | 180 | 1.883 ± 0.100 | 0.043 ± 0.004 | | 180 | 0.286 ± 0.028 | 0.221 ± 0.021 |
| | 570 | 1.199 ± 0.066 | 0.018 ± 0.001 | | 570 | 0.140 ± 0.006 | 0.100 ± 0.013 |
| | 1020 | 1.150 ± 0.060 | N/A | | 1020 | 0.081 ± 0.005 | N/A |
| blood | 2 | 18.247 ± 1.138 | 15.013 ± 0.506 | sm. int. | 2 | 3.690 ± 0.250 | 3.120 ± 0.100 |
| | 10 | 2.265 ± 0.245 | 2.047 ± 0.195 | | 10 | 7.010 ± 0.070 | 6.440 ± 0.250 |
| | 60 | 0.112 ± 0.003 | 0.102 ± 0.019 | | 60 | 15.716 ± 2.036 | 14.649 ± 0.532 |
| | 180 | 0.032 ± 0.001 | 0.034 ± 0.003 | | 180 | 1.479 ± 0.710 | 1.243 ± 0.405 |
| | 570 | 0.013 ± 0.001 | 0.011 ± 0.001 | | 570 | 0.219 ± 0.147 | 0.159 ± 0.067 |
| | 1020 | 0.006 ± 0.001 | N/A | | 1020 | 0.280 ± 0.243 | N/A |
| lung | 2 | 8.970 ± 1.210 | 8.130 ± 0.960 | lg. int. | 2 | 2.340 ± 0.240 | 2.280 ± 0.180 |
| | 10 | 2.130 ± 0.080 | 1.940 ± 0.230 | | 10 | 0.890 ± 0.040 | 0.770 ± 0.070 |
| | 60 | 0.157 ± 0.011 | 0.120 ± 0.005 | | 60 | 10.799 ± 5.381 | 21.655 ± 11.676 |
| | 180 | 0.048 ± 0.006 | 0.041 ± 0.003 | | 180 | 26.182 ± 7.839 | 18.023 ± 3.485 |
| | 570 | 0.028 ± 0.006 | 0.017 ± 0.002 | | 570 | 1.263 ± 0.706 | 0.716 ± 0.179 |
| | 1020 | 0.007 ± 0.001 | N/A | | 1020 | 0.298 ± 0.167 | N/A |
| liver | 2 | 9.120 ± 0.530 | 7.900 ± 0.350 | muscle | 2 | 1.270 ± 0.130 | 1.490 ± 0.050 |
| | 10 | 12.460 ± 1.250 | 9.100 ± 0.830 | | 10 | 0.870 ± 0.090 | 1.840 ± 1.000 |
| | 60 | 1.234 ± 0.091 | 0.423 ± 0.095 | | 60 | 0.064 ± 0.003 | 0.050 ± 0.004 |
| | 180 | 0.401 ± 0.084 | 0.211 ± 0.059 | | 180 | 0.016 ± 0.002 | 0.011 ± 0.001 |
| | 570 | 0.104 ± 0.017 | 0.058 ± 0.003 | | 570 | 0.011 ± 0.002 | 0.007 ± 0.001 |
| | 1020 | 0.075 ± 0.003 | N/A | | 1020 | 0.003 ± 0.0003 | |
| spleen | 2 | 5.100 ± 0.410 | 4.860 ± 0.130 | | | | |
| | 10 | 2.460 ± 0.210 | 1.220 ± 0.120 | | | | |
| | 60 | 0.643 ± 0.076 | 0.110 ± 0.015 | | | | |
| | 180 | 0.198 ± 0.026 | 0.038 ± 0.005 | | | | |
| | 570 | 0.062 ± 0.004 | 0.020 ± 0.001 | | | | |
| | 1020 | 0.030 ± 0.003 | N/A | | | | |

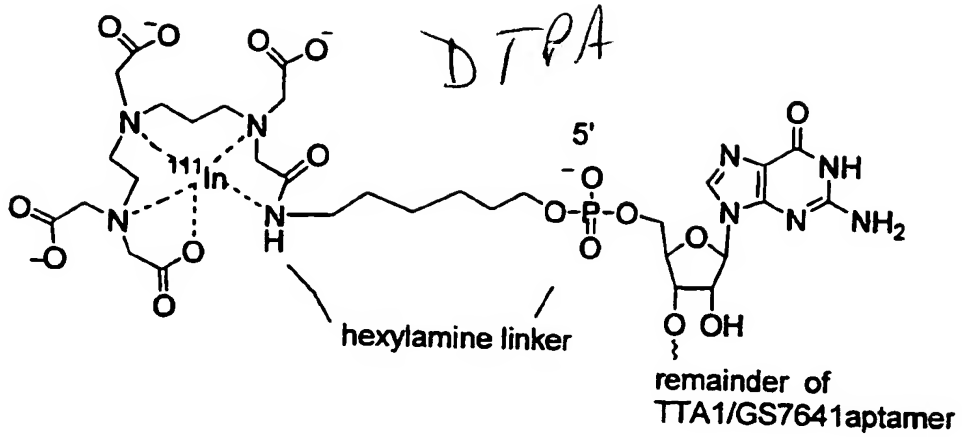


FIGURE 8

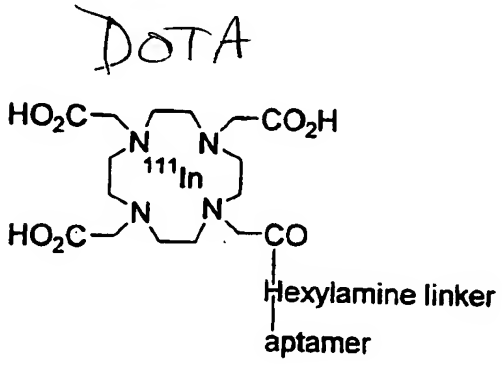


FIGURE 9

(CH2CH2O)6 linker
hexylamine linker
Delete "CCCUG" for TTA1.NB
SEQ ID NO:64
● = 2'-OH G

FIGURE 2

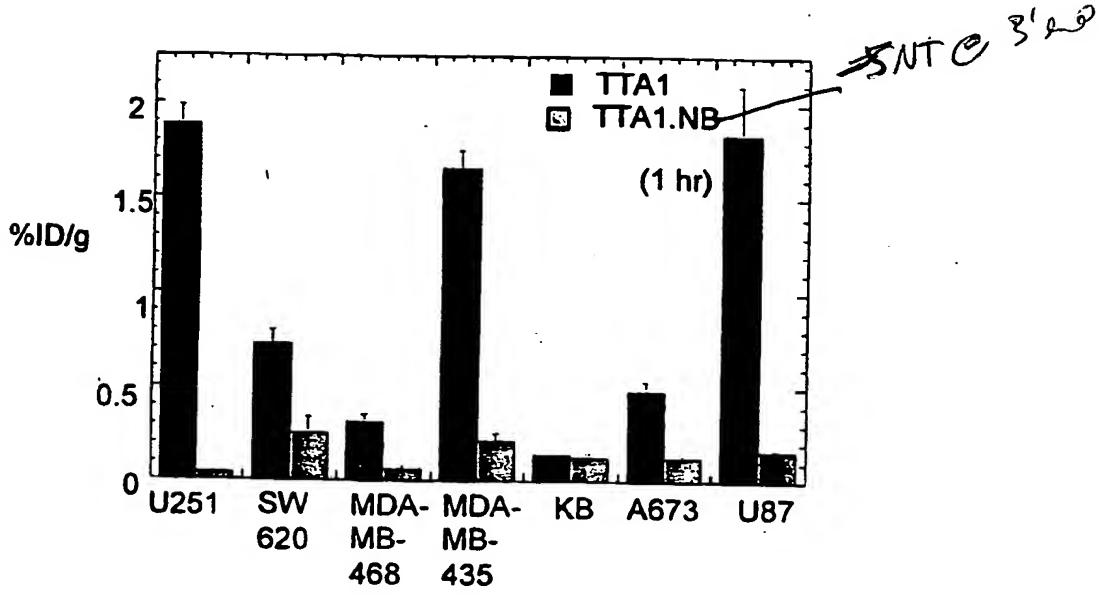


FIGURE 6

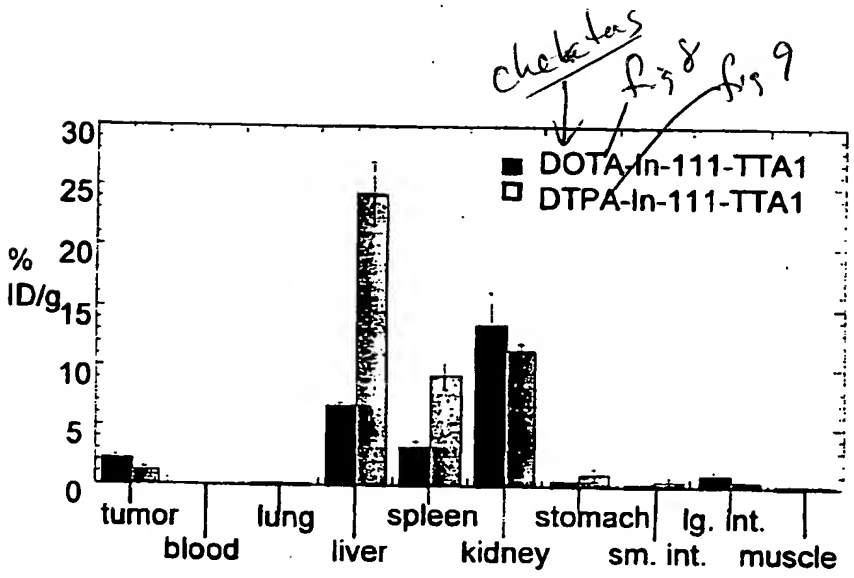


FIGURE 7

# Tenascin Expression in Cancer Cells and Stroma of Human Breast Cancer and Its Prognostic Significance[1]

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## ABSTRACT

**Sections of formalin-fixed, paraffin-embedded tissues from 210 human breast cancers were immunohistochemically examined using the mAb against human tenascin (TN) RCB1. Immunoreactive TN was detected in the breast cancer stroma in 77 (36.7%) cases, whereas the remaining 133 (63.3%) were negative. Of the 77, 12 (5.7%) cases also showed positive staining in the carcinoma cell cytoplasm. The positive cells were often observed in the margin of the cancer nests at the site adjacent to the stroma. According to the staining pattern of TN, the breast cancer cases were classified into the three groups of cancer cell TN(+)/stromal TN(+), cancer cell(−)/stromal TN(+), and cancer cell(−)/stromal TN(−). Analysis of the relationship of these TN patterns with various clinicopathological characteristics of the tumors and the patient outcome revealed that, in comparison to the cancer cell(−)/stromal TN(−) group, the cancer cell TN(+)/stromal TN(+) group exhibited increased frequency of lymph node metastasis and exceptionally poor outcome, and the cancer cell(−)/stromal TN(+) group also showed more frequent metastasis and poorer outcome. Most of the cancer cell TN(+)/stromal TN(+) cases were c-*erb*B-2 positive and estrogen receptor negative. Furthermore, *in situ* hybridization of freshly obtained breast cancer tissues demonstrated that both cancer cells and stromal cells express TN mRNA. These results indicate that the TN in breast cancer is produced by cancer epithelial cells as well as by stromal mesenchymal cells, and that cancer cell TN might be involved in cancer spreading, resulting in unfavorable patient prognosis.**

## INTRODUCTION

TN[3] is an extracellular matrix glycoprotein with a unique six-armed macromolecular structure, which is known to be an essential factor for modulation of reciprocal interactions between the epithelium and mesenchyme during embryogenesis (1–3). A number of studies have demonstrated prominent TN expression in human cancers including those of brain (4), colon (5, 6), liver (7, 8), lung (9, 10), uterus (11), skin (12), prostate (13), and mammary gland (14–17). Because it was initially proposed as a stromal marker for epithelial malignancy (14), TN expression in cancers has usually been observed in the stroma. Examining human breast cancers by immunohistochemistry, investigators have found that TN is consistently present in the stroma of malignant tumors (14–16). Our previous examination also indicated intense staining in the connective tissue of invasive ductal carcinomas (17). Accordingly, it has been generally accepted that TN is produced by the mesenchyme and has an active function in cancer development, probably by promoting cancer cell proliferation and invasion.

Shoji *et al.* (18) proposed TN to be a marker useful in predicting the survival of breast cancer patients. They investigated immunoreactive TN in 82 patients with primary invasive breast carcinomas, and found a significantly superior outcome during the 5-year period after surgery in the TN-positive patients compared with the negative patients. Immunohistochemistry of primary colon carcinomas demonstrated no lymphogenous metastasis in patients in whom the cancer stroma showed strongly positive staining for TN (19). Taking these results into consideration, it is conceivable that TN may prevent rather than enhance the cancer cell outgrowth, probably by creating a barricade surrounding the cancer nests and inhibiting the movement of these cells. In fact, TN has been proposed as a boundary molecule in the somatosensory cortical barrel field during the development of mouse cerebral cortex (20). Thus, there are two different lines of speculation as to the role of TN in cancer development.

Ligthner *et al.* (21) have recently demonstrated that normal mammary epithelial cells express TN in culture and incorporate the protein into the underlying matrix. They have also examined human breast tissues by *in situ* hybridization and found that both normal and malignant mammary epithelial cells can express TN mRNA (21). Our previous findings have shown that A431 epidermal cancer cells which express no TN in culture can produce TN, with accumulation in the surrounding mesenchyme, when injected into the nude mouse subcutis (22). Thus, the TN in the breast cancer stroma is considered to be originated from both the epithelium and mesenchyme. We have proposed that TN in the tissue is heterogeneous in its structure and function (23). In breast cancer, the structure and function of epithelial TN are possibly different from those of the mesenchymal TN. Therefore, it is important to determine by which cells the TN in the cancer stroma was produced.

In this study, we used archival formalin-fixed and paraffin-embedded surgical materials of human breast cancers. In immunohistochemical studies of cancers using anti-TN antibody, we have found positive staining in the cytoplasm of cancer cells. Therefore, this study was designed to investigate the presence of both the cancer cell TN and stromal TN in breast cancer, and the implications regarding the relationship between the TN staining

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[1] This research was supported in part by Grants-In-Aid from the Ministry of Education, Science and Culture, Japan.
[2] To whom requests for reprints should be addressed.
[3] The abbreviations used are: TN, tenascin; ER, estrogen receptor; TBS, Tris-buffered saline; DIG, digoxygenin.

# TENASCIN-C NUCLEIC ACID LIGANDS

## FIELD OF THE INVENTION

Described herein are high affinity nucleic acid ligands to tenascin-C. Also described herein are methods for identifying and preparing high affinity nucleic acid ligands to tenascin-C. The method used herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment. Further disclosed are high affinity nucleic acid ligands to tenascin-C. Further disclosed are RNA ligands to tenascin-C. Also included are oligonucleotides containing nucleotide derivatives chemically modified at the 2'-positions of the purines and pyrimidines. Additionally disclosed are RNA ligands to tenascin-C containing 2'-F and 2'OMe modifications. The oligonucleotides of the present invention are useful as diagnostic and/or therapeutic agents.

## BACKGROUND OF THE INVENTION

Tenascin-C is a 1.1-1.5 million Da, hexameric glycoprotein that is located primarily in the extracellular matrix. Tenascin-C is expressed during embryogenesis, wound healing, and neoplasia, suggesting a role for this protein in tissue remodeling (Erickson and Bourdon (1989) *Ann Rev Cell Biol* 5:71-92). Neoplastic processes also involve tissue remodeling, and tenascin-C is over-expressed in many tumor types including carcinomas of the lung, breast, prostate, and colon, astrocytomas, glioblastomas, melanomas, and sarcomas (Soini *et al.* (1993) Am J Clin Pathol 100(2):145-50; Koukoulis *et al.* (1991) Hum Pathol 22(7):636-43; Borsi *et al.* (1992) Int J Cancer 52(5):688-92; Koukoulis *et al.* (1993) J Submicrosc Cytol Pathol 25(2):285-95; Ibrahim *et al.* (1993) Hum Pathol 24(9):982-9; Riedl *et al.* (1998) Dis Colon Rectum 41(1):86-92; Tuominen and Kallioinen (1994) J Cutan Pathol 21(5):424-9; Natali *et al.* (1990) Int J Cancer 46(4):586-90; Zagzag *et al.* (1995) Cancer Res 55(4):907-14; Hasegawa *et al.* (1997) Acta Neuropathol (Berl) 93(5):431-7; Saxon *et al.* (1997) Pediatr Pathol Lab Med 17(2):259-66; Hasegawa *et al.* (1995) Hum Pathol 26(8):838-45). In addition, tenascin-C is overexpressed in hyperproliferative skin diseases, e.g. psoriasis (Schalkwijk *et al.* (1991) Br J Dermatol 124(1):13-20), and in atherosclerotic lesions (Fukumoto *et al.* (1998) *J* Atheroscler Thromb 5(1):29-35; Wallner *et al.* (1999) Circulation 99(10):1284-9). Radiolabeled antibodies that bind tenascin-C are used for imaging and therapy of tumors in clinical settings (Paganelli *et al.* (1999) Eur J Nucl Med 26(4):348-57; Paganelli *et al.* (1994) Eur J Nucl Med 21(4):314-21; Bigner *et al.* (1998) J Clin Oncol